THE RESISTANCE OF INSECTS TO PLANT PROTEINASE INHIBITORS

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Bibliographic abstract

The research reported in this thesis describes the induction of proteinase inhibitor synthesis in solanaceous plants (tobacco and tomato), when lepidopteran larvae (*Manduca sexta* and *Spodoptera exigua*) are feeding on leaves. It is shown that the larvae circumvent the proteinase inhibitor defense of these plants by the induction of non-susceptible gut proteinases. A phage display method is presented, which may allow the isolation of PIs that are also active against the non-susceptible proteinases of insects. It is expected that the application of such PIs can complement the natural PI defense of plants, and result in the protection of transgenic plants against insects.

Key-words: proteinase inhibitors, insect resistance, transgenic plants, phage display

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STELLINGEN

1. Een proteïnase remmer kan voor insekten toxisch zijn in de ene, maar zonder effect in de andere plantesoort.

   Dit proefschrift

2. De ongevoeligheid van insekten voor de proteïnase remmers van hun waardplant bewijst het belang van deze eiwitten voor de bescherming van planten.

   Dit proefschrift

3. De synthese van proteïnase remmers is wond-induceerbaar om de kosten te drukken.

   Dit proefschrift

4. Johnson *et al.* (1989) en McManus *et al.* (1994) hebben niet bewezen, dat de expressie van proteïnase remmers in transgene tabaksplanten resistentie tegen lepidopteren geeft.

   Johnson *et al.* (1989) Proc. Natl. Acad. Sci. USA 86: 9871-9875
   McManus *et al.* (1994) Transgenic Res. 3: 50-58

5. De conclusies van de artikelen van Broadway en Duffey (1986) en Broadway (1995) over het resistentiemechanisme van proteïnase remmers in lepidopteren zijn gebaseerd op artefacten.

   Broadway and Duffey (1986) J. Insect Physiol. 32: 827-833
   Broadway (1995) J. Insect Physiol. 41: 107-116

6. Proteïnase remmers, die in concentraties van 1-10% (w/v) in artificiële dieten de ontwikkeling en overleving van insekten remmen, zijn waarschijnlijk niet van nut voor toepassingen in transgene planten gericht tegen deze insekten.

   Gatehouse *et al.* (1979) J. Sci. Food Agric. 30: 948-958
   Gatehouse *et al.* (1983) J. Sci. Food Agric. 34: 345-350

7. Het is efficienter om de synthese van proteïnases te remmen dan de proteïnases zelf.

8. De door Mattiaci *et al.* (1994, 1995) veronderstelde betrokkenheid van de β-glucosidase elicitor in het speeksel van *Pieris brassicae* larven op kool bij het aantrekken van *Cotesia glomerata* sluipwespen is voorbarig, omdat hun experimenten niet uitsluiten dat mechanische rupseschade alleen een voldoende reden voor aantrekking is.

Mattiacci *et al.* (1994) J. Chem. Ecol. 20: 2229-2247
Mattiacci *et al.* (1995) Proc. Natl. Acad. USA 92: 2036-2040

9. De voorspelbaarheid van het succes van genetische modificatie van planten wordt groter, naarmate het ingebrachte gen minder verschilt van wat reeds in het genoom aanwezig is.

10. Een evolutietheorie komt niet uit de hemel vallen en hoort daarom in het eindexamen thuis.

Stellingen behorende bij het proefschrift getiteld
"The resistance of insects to plant proteinase inhibitors",
door M.A. Jongsma, in het openbaar te verdedigen
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ABSTRACT

The research reported in this thesis describes the induction of proteinase inhibitor synthesis in solanaceous plants (tobacco and tomato), when lepidopteran larvae (*Manduca sexta* and *Spodoptera exigua*) are feeding on leaves. It is shown that the larvae circumvent the proteinase inhibitor defense of these plants by the induction of non-susceptible gut proteinases. A phage display method is presented, which may allow the isolation of PIs that are also active against the non-susceptible proteinases of insects. It is expected that the application of such PIs can complement the natural PI defense of plants, and result in the protection of transgenic plants against insects.

Chapter one provides a general introduction to plant PIs. It describes the different PI families identified in plants, their mode of action against serine proteinases and the available evidence for a defensive role in plants. The effects of dietary PIs in insects are reviewed, and the physiological mechanisms resulting in growth depression in vertebrates are discussed. Chapter two presents a simple, but powerful method to measure quantitatively activities of a wide range of serine proteinase inhibitors using a radial diffusion assay. The assay can detect as little as 2-20 pmol PI, the error is between 4-12%, and the detection range can vary by three orders of magnitude. In chapter three the induction of endogenous PIs in response to insect attack is compared to the response after mechanical wounding and virus infection. It is demonstrated that local induction of PIs after insect attack is very strong in mature tobacco and tomato plants, but that systemic induction is virtually absent. Instead of direct systemic PI induction, it is observed that wounding several leaves at once, creates locally a stronger wound response. This suggests the presence of a systemic factor, which regulates the strength of the local wound response by silent alarm. Chapter four describes the induction of proteinase activity insensitive to plant PIs in the gut of *Spodoptera exigua* larvae, when the insects are feeding on tobacco leaves containing either potato PI2 or endogenous tobacco PIs. It is demonstrated that PIs decrease the proteinase activity in larval guts, but that this reduction is partially compensated for by the induction of PI-insensitive proteinase activity. The weight of larvae, fed with PI leaves, was not reduced, so that the induced PI-insensitive activity, apparently sufficiently, compensated proteinase activity lost by inhibition. Chapter five describes the analysis of gut proteinase activity of *S. exigua* larvae. Six major proteinase activities were identified and three were purified by anion exchange chromatography and further analyzed. One of the purified proteinases was a cysteine proteinase with optimal activity at pH 11 and is the first example of this class of proteinase to be isolated from a lepidopteran insect. Chapter six demonstrates that potato PI2 can be displayed as a functional protein on M13-phages by fusion to a minor coat protein. It is shown that functional PI2-phages mixed with non-functional phages can be enriched 323,000-fold against trypsin after three selection rounds. Large engineered phage libraries of PI2-variants allow the selection of PI2 clones with high affinity for PI-insensitive proteinases of insect pests. Finally, chapter seven discusses the results comprehensively to defend the thesis that insects acquire resistance against the PIs induced in their host plants. It is argued that the successful application of PIs for resistance breeding will require the selection of better PI genes and that phage display offers a suitable method for this purpose.

CONTENTS

LIST OF ABBREVIATIONS

α1-AT	human α1-Antitrypsin
APNE	N-Acetyl-DL-phenylalanine-β-naphtyl ester
ABA	Abscisic acid
ATP	Adenosine triphosphate
BANA	Nα-Benzoyl-DL-arginine-β-naphthylamide
BA*p*NA	Nα-Benzoyl-L-Arg-*p*-nitroanilide
BTEE	N-Benzoyl-L-tyrosine-ethyl-ester
CCK	Cholecystokinin
CpTI	Cowpea trypsin inhibitor
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
E-64	Trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane
ELISA	Enzyme-linked immuno-sorbent assay
FPLC	Fast protein liquid chromatography
HPLC	High pressure liquid chromatography
IAA	Iodoacetic acid
kDa	Kilodalton
M_r	Relative molecular weight
MM	*Minimal medium*
MW	Molecular weight
NEM	N-Ethyl-maleimide
OD	Optical density
OVOI	Ovoinhibitor from chicken egg white
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pHMB	*p*-Hydroxy-mercuribenzoic acid
PI	Proteinase inhibitor
PI1	Potato proteinase inhibitor I
PI2	Potato proteinase inhibitor II
PMSF	Phenylmethylsulfonyl fluoride
*p*NA	*p*-Nitroaniline
PR	Pathogenesis-related
RNA	Ribonucleic acid
SAAPL*p*NA	N-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide
SBBI	Soybean bowman birk inhibitor
SDS	Sodium dodecylsulphate
SI	Subtilisin inhibitor
SPI	Serine proteinase inhibitor
STI	Soybean trypsin inhibitor
TCA	Trichloro-acetic acid
TI	Trypsin inhibitor
TMV	Tobacco mosaic virus

ACCOUNT

Parts of this thesis have been included in the following publications:

Jongsma, M.A., Bakker, P.L., Stiekema, W.J. (1993) Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. *Analytical Biochemistry* **212**: 79-84

Jongsma, M.A., Bakker, P.L., Visser, B., Stiekema, W.J. (1994) Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* **195**: 29-35

Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D., Stiekema, W.J. (1995) Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of proteinase activity insensitive to inhibition. *Proceedings of the National Academy of Sciences of the USA* **92**: 8041-8045

Jongsma, M.A., Peters, J., Bosch, D., Stiekema, W.J.: Characterization and partial purification of gut cysteine and serine proteinases of *Spodoptera exigua* Hübner (Lepidoptera: noctuidae). *Insect Biochemistry and Molecular Biology* (accepted with revisions)

Jongsma, M.A., Bakker, P.L., Stiekema, W.J., Bosch, D. (1995) Phage display of a double-headed proteinase inhibitor: analysis of the binding domains of proteinase inhibitor II. *Molecular Breeding* 1: 181-191

Bolter, C.J., Jongsma, M.A.: Colorado potato beetles adapt to proteinase inhbitors induced in potato leaves by methyl jasmonate. *Journal of Insect Physiology* (in press)

CHAPTER 1

GENERAL INTRODUCTION

Proteinase inhibitors (PIs) of plants are among the best studied proteins in plant biochemistry and biology. The elucidation of the three-dimensional structures of several plant PIs in complex with proteinases has provided a detailed understanding of their mode of action. PI genes are induced by environmental stress, pathogen or insect attack and developmental factors, and the dissection of the pathways resulting in PI gene transcription is presently a major area of signal transduction research. The effects of ingested PIs on insect growth and development have been seriously investigated for more than 15 years. These studies have established the role of PIs in plant defense against insects, but a detailed understanding of the mechanism of action has not yet been achieved. This introduction will briefly introduce the different proteinases and plant PIs that have been characterized in nature and discuss the evidence for their defensive role in plants. The developmental delay that ingested PIs cause in insects is discussed in relation to the proposed mechanisms of action in vertebrates. It is proposed that understanding the mechanism of action of PIs in insects will allow a better use of PIs in crop protection against insects.

Occurrence and roles of plant proteinase inhibitors

There are four known classes of proteinases, which are distinguished on the basis of the central amino acid residue (serine, cysteine, aspartate) or metal ion involved in catalyzing cleavage of peptide bonds in protein substrates (Hartley, 1970; James, 1976; Barrett, 1986). The number of different proteinase gene families is strictly limited. For example, only two different gene families of serine proteinases, the chymotrypsin and subtilisin superfamily, are found in nature. The proteinaceous inhibitors of these proteinases are a much more diverse group of proteins than their proteinase counterparts. Bacteria, fungi, animals and plants each have a range of inhibitor gene families unique for these types of organisms. They are mostly classified on the basis of gene family relationships, because the spectrum of activities of PIs can be very broad, while at the same time a single amino acid difference can result in a completely different specificity. Determination of the crystal structures in complex with proteinases has allowed a deeper understanding of the different mechanisms of proteinase inhibition by PIs (Bode and Huber, 1994). Some PIs (hirudin, cystatin) are tight, reversible binders that do not directly contact the substrate binding site of the proteinase. Instead, they overlap it, and thereby sterically inhibit enzymatic activity. Most PIs, however, obey the standard mechanism and can be regarded as highly specific substrates of proteinases. They are inhibitors, because hydrolysis of the peptide bond is very slow and incomplete, while the hydrolyzed PI is still capable of strongly binding to the proteinase substrate binding site. Tight binding is the result of multiple van der Waals' and electrostatic (hydrogen bonds) interactions between proteinase amino acid residues around the substrate binding site and inhibitor amino acid residues around the peptide bond to be hydrolyzed (see also chapter 7). Hydrolysis of the peptide bond does not disturb these

interactions because the scaffold inhibitor protein and disulphide bridges uphold the peptide domain in its original conformational structure. An example of an inhibitor-proteinase complex interacting by the standard mechanism is shown in Fig. 1 for proteinase inhibitor II (PI2).

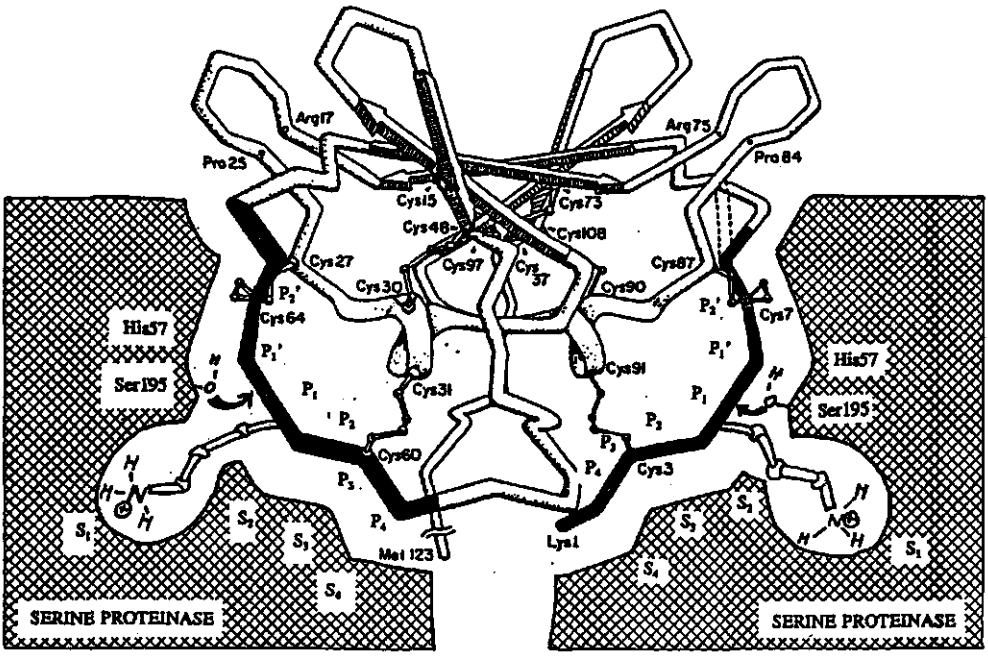

Figure 1. The proposed crystal structure of the double headed proteinase inhibitor II molecule (taken from Greenblatt *et al.*, 1989). The arrow indicates the attack of the hydroxyl group of Ser195 on the P_1-P'_1 amide bond of the inhibitor. The two proteinase molecules (cross-hatched) are inhibited because the inhibitor loops in solid black directly contact the proteinase molecules in such a way that residues P_6 to P_4' bind to the corresponding substrate binding sites S_6 to S_4'. The sidechain of the P_1-residue is drawn to illustrate its important interaction with the proteinase specificity pocket, S_1. The substrate binding sites can represent more than one residue (see also chapter 7).

Plant proteinase inhibitors occur in storage organs like seeds and tubers, in reproductive organs, and in vegetative cells of virtually all plant families that have been investigated (Garcia-Olmedo *et al.*, 1987; Ryan, 1990; Richardson, 1991). In these tissues PIs have been found as crystals in the cytoplasm (Rodis and Hoff, 1984), as dense protein aggregates in the vacuole (Wingate *et al.*, 1991), and as secreted proteins in the intercellular fluid (Cornelissen *et al.*, 1986). Table 1 lists the different PI gene families presently known in plants and subdivides them on the basis of inhibitory acitivity towards the different classes of proteinases. Most inhibitors are specific for only one class of proteinase, but the Kunitz family exhibits exceptional diversity with some members inhibiting serine or cysteine proteinases, while others are bifunctional for both serine and aspartic proteinases. A general characteristic of PIs is that they are small, cysteine-rich and heat-resistant proteins of 3-23 kDa (excluding tandemly repeated inhibitor domains of 36-80 kDa). Chemical reduction of

Table 1 Plant proteinase inhibitor families

Family	Monomer Mr[1] (kD)	Monomer 1/2 Cys[2]	# active domains[3]	Inhibited Enzymes[4]	Occurence[5]	References
Serine proteinase inhibitors						
Cucurbit	3	6	1	T,H	Cucurbitaceae	Hojima *et al.*, 1980 Wieczorek *et al.*, 1985
Potato Inhibitor I	8-9 (40)	0,2	1	T,C,S	Solanaceae Gramineae Polygonaceae Leguminosae Cucurbitaceae *Hirudo medicinalis*[6]	Melville and Ryan, 1970 Plunkett *et al.*, 1982 Seemüller *et al.*, 1980
Potato Inhibitor II	6,12,18,36	8,16,24,48	2,3,6	T,C,S	Solanaceae	Richardson *et al.*, 1979 Hass *et al.*, 1982 Graham *et al.*, 1985
Bowman Birk	8-9 (14)	14,18	2,4	T,C,E	Leguminosae Gramineae	Tan and Stevens, 1971 Odani *et al.*, 1986 Tashiro *et al.*, 1987
Cereal superfamily	12-13	10	1 (B)	T,H,(A)	Gramineae	Kashlan and Richardson, 1981
Kunitz	21-22	4	1(B)	T,C,S,K,CP (CD,A)	Leguminosae Gramineae Araceae Alismataceae	Koide and Ikenaka, 1973a,b Yoshikawa *et al.*, 1976 Svendsen *et al.*, 1986
Thaumatin-PR like	22-23	16	1 (B)	T,(A)	Gramineae Solanaceae	Blanco-Labra and Iturbe Chinas, 1981 Richardson *et al.*, 1987 Edens *et al.*, 1982 Cornelissen *et al.*, 1986
Cysteine proteinase inhibitors						
Cystatin superfamily	12,80	0	1,8	CP	Gramineae Solanaceae animals	Abe *et al.*, 1987 Walsh *et al.*, 1993
Potato CPI 8.3 [Kunitz family]	20	4	1	CP	Solanaceae	Krizaj *et al.*, 1993
Aspartic proteinase inhibitors						
Cathepsin D Inh. [Kunitz family]	20	4	1 (B)	CD,(T)	Solanaceae	Mares *et al.*, 1989 Ishikawa *et al.*, 1994a,b Strukelj *et al.*, 1992
Metallo-proteinase inhibitors						
Carboxypeptidase inhibitors	4	6	1	CaP	Solanaceae	Hass *et al.*, 1975,1976

[1] Between brackets the native molecular mass is indicated

[2] The number of half-cysteines present in an inhibitor monomer

[3] The number of active domains as the result of tandem gene duplication, between brackets inhibitors with more than one non-homologous functional domain are indicated by (B)

[4] Inhibited enzymes: trypsin (T), chymotrypsin (C), elastase (E), subtilisin (S), kallikrein (K), Hageman factor (H), cysteine proteinases (CP), cathepsin D (CD), amylase (A), Carboxypeptidase (CaP). Between brackets are bifunctional activities indicated.

[5] The listed plant families reflect known sources but do not exclude any other plant family.

[6] Eglin C from leech (animal source)

disulphide bridges generally leads to the loss of inhibitory activity, suggesting an important function of these disulphides in the stability and active conformational structure of the proteins. A remarkable feature of these proteins is that many are multi-headed in some way. Some of them are bifunctional and have two heterologous domains that inhibit two different enzymes (Kunitz inhibitor family, Cereal superfamily, Thaumatin/PR-like family). Others form multimers of homologous domains either post-translationally (PI1, PI2, Bowman Birk inhibitor family) or by a tandemly repeated gene structure (PI2, Bowman Birk inhibitor family, multicystatin). The functional advantage of multimers is not clear, but they may increase the stability of the proteins by reducing the surface area exposed to gut proteinases, or they may create an avidity effect similar to antibodies.

The inhibitory activities of proteinaceous plant proteinase inhibitors are directed against a wide range of prokaryotic and eukaryotic proteinases, (reviews: Ryan, 1973, 1978, 1990; Richardson, 1977, 1991). Roles in regulating plant developmental processes like germination have been postulated (reviewed by Ryan, 1973), but in most cases the endogenous plant proteinases are not inhibited by these inhibitors, and even degrade them (Papastoitsis and Wilson, 1990). Potato multicystatin, a cysteine proteinase inhibitor, accumulates as crystals in the cytoplasm physically separated from vacuolar proteinases (Rodis and Hoff, 1984; Walsh *et al.*, 1993), and these proteinases are insensitive to this type of inhibitor (Michaud *et al.*, 1994). This suggests that plants have evolved some proteinase inhibitors specifically for activity against proteinases of pests or pathogens. Indeed, the multicystatin crystals solubilize at the acid pH of guts of insects that utilize cysteine proteinases for protein digestion and were demonstrated to affect growth of *Diabrotica* larvae when incorporated in artificial diets (Orr *et al.*, 1994).

Other evidence for the role of PIs in plant defense can be found in the way PIs are regulated in tomato fruit of the wild tomato species, *Lycopersicon peruvianum*. Tomato seeds are dispersed by animals that eat the red ripe fruit containing low PI levels. To prevent insects and animals from eating the equally nutritious, but unripe fruits containing immature seeds, the taste and colour is different (green). In addition, the fruits contain exceedingly high PI levels (up to 50% of total protein) that are actively broken down upon ripening (Pearce *et al.*, 1988). The correlation of the dispersal mode of plant species with the PI levels at different stages of fruit development was subsequently examined for a large number of seeds dispersed passively or by animals or wind (Hebbar *et al.*, 1993). The animal dispersed seeds had very high PI levels during fruit ripening, but not at the ripened stage, while wind and passive dispersed seeds had low levels throughout seed development, confirming the role of PIs in preventing premature predation.

The role of PIs in plant defense against insects was demonstrated more directly when Green and Ryan (1972) showed the induction of PIs (PI1 and PI2) in wounded and non-wounded leaves of potato in response to wounding and insect feeding. In subsequent experiments also other PIs (Bowman Birk inhibitor, Kunitz inhibitor) in other plants species (poplar, alfalfa) were found to be systemically induced by wounding (Plunkett *et al.*, 1982; Nelson *et al.*, 1983; Brown and Ryan, 1984; Bradshaw *et al.*, 1989; Davis *et al.*, 1991). The systemic induction of PIs in non-wounded leaves in response to mechanical damage and insect feeding suggests that plants possess a mechanism of defense functioning to protect them against chewing predators like insects or vertebrates. The relatively slow induction of PI activity may restrict the effectiveness of this defense mainly to slow leaf feeders like insects. The signal leaving the wound site and initiating the signal transduction leading to PI gene transcription was demonstrated to travel at the speed of assimilates (35-120 mm/min)

through the phloem (Nelson *et al.*, 1983). Originally, oligosaccharides of a certain chain length were assumed to be the systemic messengers (Bishop *et al.*, 1981; 1984; reviewed by Ryan and Farmer, 1991), but Baydoun and Fry (1985) showed that these oligosaccharides were mobile through xylem vessels, but not through phloem tissue via which the wound signal is presumably transported *in vivo*. Other compounds that have since been suggested to act as systemic signal molecules include abscisic acid, and an 18-amino acid polypeptide called systemin, but also action potentials and changes in hydrostatic pressure have been claimed to have a role in signalling wounding events (reviewed by Ryan, 1992). The role of abscisic acid (ABA) was investigated with ABA-deficient mutants of potato (Peña-Cortés *et al.*, 1989). In these mutants PI-transcripts were only induced by supplying ABA through the petioles or by spraying ABA on the leaves and not by wounding. In addition, ABA levels in normal plants were significantly increased by wounding in both wounded and non-wounded leaves. There has been no direct demonstration, however, that ABA acts as the systemic messenger. It may equally well act more downstream of the signalling pathway (Farmer and Ryan, 1992; Peña-Cortés, 1994). Wildon *et al.* (1992) claimed that systemic induction of PI genes upon wounding was correlated with action potentials and did not require a phloem-translocated chemical signal. Effects of externally applied action potentials without wounding were not demonstrated, however, and systemic signalling was only observed with wounded cotyledons and not with normal leaves (Bowles, 1994). Systemin is presently the most interesting remaining candidates for systemic signalling (Pearce *et al.*, 1991). Recently, it was shown that plants overexpressing the gene encoding the 200-amino acid precursor of systemin (prosystemin) resulted in high constitutive levels of serine proteinase inhibitors, demonstrating the powerful ability of the gene product to induce high levels of PI activity *in vivo*. More importantly, however, grafting of wildtype scions on the overexpressing rootstocks resulted in constitutive expression in the scions as well. This demonstrated *in vivo* that systemin can act as a systemic messenger (McGurl *et al.*, 1994a,b). In this instance wounding was possibly not necessary to release the signal, because the 35S CaMV promoter also drives the expression of genes in phloem tissue which is responsible for the translocation of the wound signal.

Effects of dietary plant proteinase inhibitors in insects

The effects of purified plant PIs on insect growth and development have been investigated by rearing insects on artificial diets supplemented with these inhibitors. The effectiveness of a proteinase inhibitor then depends upon several factors, including its potency to reduce proteinase activity, the resistance of the inhibitor to breakdown by gut proteinases and the amino acid composition and digestibility of the protein contained in the diet (Broadway and Duffey, 1986a; Burgess *et al.*, 1991, 1994; Hinks *et al.*, 1991). When added to diets in concentrations of 0.1-10% (approximately 0.1-10 mM!), proteinase inhibitors retarded the growth of several lepidopteran insects (Broadway and Duffey, 1986b; Broadway and Colvin, 1992; Steffens *et al.*, 1978; Larocque and Houseman, 1990; Hilder *et al.*, 1990; Johnston *et al.*, 1993; Shukle and Murdock, 1983), coleopteran insects (Hines *et al.*, 1990; Murdock *et al.*, 1988; Wolfson and Murdock, 1987; Gatehouse *et al.*, 1979; Gatehouse and Boulter, 1983; Oppert *et al.*, 1993; Birk and Applebaum, 1960; Chen *et al.*, 1992; Orr *et al.*, 1994), orthopteran insects (Burgess *et al.*, 1991, 1994). In dipteran insects strong effects on fecundity, egg hatch and survival were reported (Spates, 1979; Deloach and Spates, 1980; Spates and Harris, 1984).

Conclusions regarding the effectiveness of PIs against insects drawn from studies with artificial diets are not without controversy, because often the purified samples are relatively poorly characterized or the required concentrations are exceptionally high. An extensive debate was initiated by results of Gatehouse *et al.* (1979) and Gatehouse and Boulter (1983). They reported that purified trypsin inhibitors from soybean (SBTI), lima bean (LBTI), and especially cowpea (CPTI) were effective anti-metabolites against larvae of the bruchid beetle, *Callosobruchus maculatus*, if added to artificial seeds at concentrations of 1.5-10% (w/w). These *millimolar* PI concentrations far exceeded the *micromolar* concentrations normally found in leaves and seeds, but more importantly they far exceeded the *micromolar* concentrations of proteinases in the insect gut (personal observations, chapter 4). Dissociation constants of proteinase-inhibitor complexes are generally in the nanomolar range so that dietary inhibitors should be fully effective at a micromolar concentration. The need for higher concentrations suggests that either contaminating inhibitor proteins or exceptionally poor inhibition were responsible for the inhibition of gut proteinases (Gatehouse *et al.*, 1985) and growth reduction (Gatehouse *et al.*, 1979; Gatehouse and Boulter, 1983). Presumably for these reasons, other labs were not able to confirm mortality effects with their own purified preparations (Zhu *et al.*, 1994), nor could they correlate high CpTI levels with resistant cowpea cultivars (Xavier-Filho *et al.*, 1989). It illustrates that claims of antimetabolic effects of millimolar PI concentrations in artificial diets are of little biological interest, because such protein concentrations are 10-100 fold higher than found in plants.

The advent of plant biotechnology has made it possible to study elegantly the effects of single PIs expressed in transgenic host plants on insects. Nevertheless, with transgenic plants the experimental situation is more complex compared to artificial diets, because plants respond to being eaten. As described above leaf feeding insects induce in the plant an array of defense compounds including PIs. If the transgenic PI has a different spectrum of activity compared to endogenous PIs a synergistic effect can be expected, but if the spectrum of activity is the same, and the induction of endogenous PI activity is rapid enough, a resistant phenotype may not be observable. Nevertheless, it was reported that overexpression in tobacco of a cowpea trypsin inhibitor cDNA clone (Hilder *et al.*, 1987) and a potato PI2 gene (Johnson *et al.*, 1989; McManus *et al.*, 1994a) reduced larval weight gain of *Heliothis zea* (Lepidoptera: Noctuidae), *Manduca sexta* (Lepidoptera: Sphingidae) and *Chrysodeixis eriosoma* (Lepidoptera: Noctuidae) by about 50%. These results provided direct evidence that PIs affect insect growth *in planta*. The toxicity is low, however, as development was only delayed by one or two days. The reasons for this low toxicity could have been investigated by measuring *in vivo* effects on gut proteinase activity, but unfortunately such data have not yet been published for transgenic plants.

Broadway and Duffey (1986) were the first to examine in some detail physiological effects of the inhibition of gut proteinase activity by potato PI2 and soybean trypsin inhibitor on larvae of *Heliothis zea* and *Spodoptera exigua* (Lepidoptera: noctuidae) reared on artificial diet. They reported the surprising observation that gut tryptic activities were not lowered by dietary PIs, even though *in vitro* 80% of the activity can be inhibited. They proposed that the guts of insects responded to PIs by overproducing the inhibited proteinases in analogy to what was found for vertebrates. In their concept the measured gut activity represented a surplus activity over and above a large inhibited fraction. Effects on growth (20-50% reduction) were interpreted to have been caused by overproduction of enzyme leading to depletion of essential amino acids. Recently, two other groups also measured gut activities after prolonged *in vivo* exposure to PIs (Burgess *et al.*, 1994, Orr *et al.*, 1994). When PIs

severely affected growth and survival of the insects, gut activities towards specific substrates were also severely reduced. This suggests that in these insects growth is inhibited by a direct reduction of proteolytic activity. The specific substrates used in these papers do not provide a good indication of total proteolytic activity, however, and this may explain why, with neonate larvae, activities towards specific substrates were increased (Burgess *et al.*, 1991).

The different results indicate that the mode of action of PIs in insects is complicated. Studies aiming to understand the physiological response to proteinase inhibition are urgently needed to clarify why sometimes normal or higher, and sometimes reduced levels of proteinase activity are observed after the ingestion of PIs by insects, and why the effects on growth are only moderate.

Mechanisms of effect of plant proteinase inhibitors in vertebrates

The important food crops, cereals, beans and potatoes contain high concentrations of proteinase inhibitors (10-15% of total protein), which have long been known to negatively affect the growth of young animals (Klose *et al.*, 1946; Ham *et al*, 1945; Westfall and Hauge, 1948). The growth depressing properties of PIs could be alleviated by heating, but raised interest in the underlying physiological mechanisms. This research has resulted in several theories which may also help to explain effects observed in insects (reviewed by Liener and Kakade, 1980; Gallaher and Schneeman, 1983). The relative importance of each mechanism is dependent on the diet composition, the animal species and the type of inhibitor. The different mechanisms are briefly discussed below.

The most simple and direct mechanism first proposed by Almquist and Merritt (1951, 1953) claims that the inhibition of gut proteinases reduces the digestion of dietary protein, and that, therefore, less amino acids essential for growth are available. Evidence for this mechanism was found with newly hatched chicks. Pearce *et al.* (1979, 1983) observed that feeding chicks for 10 days with potato carboxy peptidase inhibitor, potato PI2, and soybean Bowman Birk inhibitor in the diet reduced their final weights by 12-27%. The presence of inhibitors was correlated with depressed gut proteinase activities (Alumot and Nitsan, 1961; Nitsan and Alumot, 1964), and a higher protein output in chick faeces, but not with a reduced food intake suggesting that, in this case, the inhibitors were effective by reducing digestion of dietary protein.

Growth of rats and mice, that were given a diet containing predigested protein or free amino acids, was still reduced by the addition of trypsin inhibitors, even though protein digestion was not necessary anymore for the release of amino acids (Liener *et al.*, 1949; Westfall *et al.*, 1948). When it was subsequently shown that, in contrast to *in vitro* measurements, proteolytic activity was not decreased *in vivo* by these trypsin inhibitor preparations, Lyman and Lepkovsky (1957) proposed a second mechanism that growth depression is the result of the hyperactive pancreas. The pancreas responds in a compensatory fashion to the effects of trypsin inhibitor, and heavily diverts essential amino acids into a spectacular increase of the synthesis of proteinases.

A third mechanism is based on the observation that the same hormone cholecystokinin (CCK) that induces the pancreas to synthesize more proteinases also gives rise to a reduced appetite (Smith and Gibbs, 1987). Dual inhibitors of both trypsin and chymotrypsin, like PI2, stimulate the level of circulating CCK, and, indeed, human subjects that were given PI2 mixed in a soup, 10 minutes later chose to eat a meal that was 18% lower in energy (Hill *et al.*, 1990), which suggests that dietary PIs could affect growth by reduced food intake.

For determining the correct strategy in transgenic plants it appears crucial to know the relative contribution of these mechanisms to growth reduction in insects. If insects fully compensate proteinase inhibition with hyperproduction of proteinases than the aim must be to achieve the highest level of PI expression in plants. If, however, the *in vivo* reduction of gut proteolytic activity is causing growth reduction than it is more important to use more active inhibitors.

Perspective

Proteinase inhibitors are ubiquitous in plants and there is ample evidence for a role of PIs in plant defense. Nevertheless, the effects on insects are not spectacular and require high concentrations of inhibitors. With this knowledge it is surprising that so many plants exhibit this form of defense. It may be that we underestimate the ecological effects on insect populations of relatively small modifications of life history parameters, but we may also be testing the wrong kind of insects. Insects, that are presently a pest on plants, may represent the few species that have become resistant to plant PI defense. In that case, we are also witnessing the result of co-evolution of insects, to overcome this form of plant defense. These challenging questions are addressed in this thesis. It describes the events taking place in both the plant and the insect when *Spodoptera exigua* larvae feed on tobacco plants expressing high PI levels, and provides a new method to improve PI2 specifically against proteinases of *S. exigua* larvae that were purified and characterized.

CHAPTER 2

QUANTITATIVE DETERMINATION OF SERINE PROTEINASE INHIBITOR ACTIVITY USING A RADIAL DIFFUSION ASSAY

An improved, time efficient, visual assay for quantitative determination of proteinase inhibitor activity in protein extracts is reported. Proteinase inhibitor activity of mammalian, bacterial and fungal serine proteinases can be quantified. The method relies on radial diffusion of proteinase inhibitor containing extracts from a central well through an agar gel containing a serine proteinase. After an incubation period the agar gel is stained via the diazo coupling of the ß-naphtol produced by the enzymatic hydrolysis of N-acetyl-D,L-phenylalanine-ß-naphtylester. Circular zones containing inhibitor-proteinase complexes remain colorless while the region containing only proteinase shows a bright pink-purple color. A reference curve relates the diameter of the colorless zone to the logarithm of the proteinase inhibitor concentration. The error in the estimation of a proteinase inhibitor quantity varying between 10-1000 pmol is 4-12%. The sensitivity of the assay is approximately 2-20 pmol of inhibited proteinase molecules depending on the inhibitor-proteinase complex assayed. The sensitivity of the assay can be enhanced tenfold or more by dilution of the proteinase concentration in the agar and by a reduction of the agar thickness.

INTRODUCTION

Methods of radial diffusion in agar gels are commonly used in immunological applications for the rapid determination of specific protein levels in large numbers of samples (Ryan, 1967). Immunological methods for monitoring serine proteinase inhibitor (SPI) levels require that these proteins have been purified for the production of antisera. In an initial investigation of SPIs of an organism the step towards purification has often not been taken yet. First, a general survey of the properties of the SPIs is carried out. For this purpose several methods have been developed that take advantage of the ability of serine proteinase inhibitors to bind stoichiometrically and with high affinity to readily available purified serine proteinases. These methods provide important biochemical information on the type and level of activity found in an extract and in that respect are complementary to immunological methods.

When the PI level in only a few samples needs to be determined most often a spectrophotometric or titrimetric assay is used (Richardson, 1991). This is the preferred method for highly accurate determinations. A drawback from these methods is that they are time-consuming and relatively insensitive. Radial diffusion of inhibitors in agar gels offers a useful time-saving alternative to the spectrophotometric or titrimetric assays. Visualization of the inhibitors is performed by either including casein or a serine proteinase in the agar. Using agar gels containing casein a cleared zone develops after pipeting a constant amount of protease with a variable amount of inhibitor in a central well. The inhibitor concentration

is inversely proportional to the size of the cleared zone (Gallagher *et al.*, 1986; Shukle and Murdock, 1983). Using agar gels containing a proteinase, after diffusion of variable amounts of proteinase inhibitors from a well, colorless zones where the proteinase is inhibited can be developed. Visualization of these zones is achieved by using acetyl-phenylalanine-ß-naphtylester (APNE) as a substrate. The size of the zone is directly proportional to the amount of proteinase inhibitor. Kourteva *et al.* (1987) described this method in a qualitative way for detecting low level trypsin and chymotrypsin inhibitor activities in HPLC fractions. Here, the method is further developed in order to be able to accurately quantify SPI levels present in tissue extracts and to determine the specificity of these SPIs towards five different serine proteinases.

MATERIALS AND METHODS

Unless otherwise noted all chemicals were from Sigma Chemical Co., St. Louis, Mi, USA.

Preparation of the agar plates

Melted agar (Bacto-agar; Difco, Detroit, MI) solution (1.8% w/v in 0.1 M Tris-Cl buffer, pH 7.6) was cooled to 50 ^{0}C and mixed with stock solutions of proteinases to a final concentration of 42 nM active proteinase. Proteinases were from mammalian, bacterial and fungal origin: bovine trypsin (type III), bovine α-chymotrypsin (type II); subtilisin Carlsbergh (subtilopeptidase A type VIII from *Bacillus licheniformis*), pronase E (Type XIV from the bacterium *Streptomyces griseus*) and proteinase K (from the fungus *Tritirachium album* supplied by Boehringer Mannheim). The molar concentration of active enzyme was determined by measuring the extinction after a burst titration using p-nitrophenyl-p-guanidinobenzoate.HCl for trypsin (Chase and Shaw, 1967) and using N-transcinnamoylimidazole for the other enzymes (Bender *et al.*, 1966). After mixing the enzyme with the agar 100 ml or 25 ml was quickly poored in 24 x 24 cm or 12 x 12 cm square petri-dishes (from Nunc, Denmark and Greiner resp.) and allowed to solidify at 4 ^{0}C for 2-3 hours. Wells 4 mm in diameter were punched at intervals that would accommodate the expected size of the zones (1.5-3 cm). Punching was done using a hollow metal cork borer to which an aspiration apparatus was attached to remove the plugs of agar. Fresh plates were used in all cases.

Preparation of the standard inhibitor solutions

Stock solutions of 42 μM of the five following SPIs were prepared in water: Type I-S Soybean Trypsin Inhibitor (STI), Trypsin-Chymotrypsin Bowman Birk Inhibitor from Soybean (SBBI), Type IV-O purified Ovoinhibitor from chicken egg white (OVOI), human α1-Antitrypsin (α1-AT; Calbiochem), and Proteinase Inhibitor II from potato (PI-2; Calbiochem). The concentration of SPI active sites in the prepared SPI solutions was determined spectrophotometrically (8). In an Eppendorf tube 0.1 ml proteinase solution (4,2 μM active proteinase in assay buffer) and 0.5-5 μl SPI solution ($>$42 μM) were brought to a final volume of 0.25 ml with assay buffer (0.1 M Tris-Cl, pH 7.6; 10 mM $CaCl_2$) and pre-incubated for 15 min at room temperature. Subsequently, 0.5 ml azocasein solution (10 mg/ml stock in assay buffer) was added, and the mixture was incubated for 2.5 min in case

of subtilisin, 5 min in case of trypsin and 30 min in case of chymotrypsin. The reaction was stopped by adding 0.25 ml 40% TCA. After 15 min the tubes were centrifuged for 5 min at 12,000×g, and the extinction of the supernatant was measured at 340 nm in 1 ml plastic cuvettes. In all measurements the proteinase and SPI solution was omitted in the reference cuvette. The first 40% decrease in extinction would form a straight line. Graphic extrapolation of this line resulted in the volume of SPI stock solution that contained 0.1 ml × 4.2 μM enzyme = 420 pmol SPI active sites. The concentration of SPI active sites thus obtained was used to make appropriate dilutions for the standard curve.

The radial inhibitor diffusion assay

Wells were filled completely with sample solution to minimize the influence of slight variations in the thickness of the agar on the size of the inhibited zone. After diffusion at 4 °C for various times, the agar plates were rinsed once with Tris buffer (0.1 M, pH 7.6; 10 mM $CaCl_2$). A staining solution was freshly prepared: 48 mg Fast Blue B salt (Fluka, Switzerland) was predissolved in 0.5 ml water as alkaline pH reduces its solubility, then 0.1 M Tris-Cl buffer, pH 7.6 of 37 °C was added to a final volume of 90 ml, and this mixture was poured into a solution of 24 mg APNE dissolved in 10 ml N,N-dimethylformamide. The staining solution was poured into the plate to cover the agar and the plate was incubated at 37 °C for 15-60 min depending on the type of enzyme and the enzyme concentration in the agar. The solution was subsequently poured off and the dishes were rinsed with tap water. Some water was left on the agar to improve visual contrast. The diameters of the unstained inhibitor zones were estimated to the nearest 0.1 mm by eye on a horizontal illuminated screen using transparent millimeter paper. Plates were photographed on top of a lit white screen. The uncolored zones were stable for up to one year.

RESULTS

The concentrations of mixed SPI populations encountered in plant protein extracts may vary from 0.1 to 100 μM. It is required that the method allows accurate determination of these concentrations directly in a single assay. The size of an inhibited zone after a certain diffusion period depends on the time of incubation, the SPI concentration, and the molecular weight of the SPI. Experiments were designed to determine the relative influence of these parameters so that an optimal assay procedure could be formulated.

Effect of time and SPI concentration on the diameter of radial zones

The effect of the time of incubation of SPIs on the distance traveled through the agar was studied using serial dilutions of five different SPIs. The SPIs were incubated at 4 °C for 2, 4, 7, 17, 22.5, and 47 hours. The SPI concentration expressed in terms of SPI active sites (or inhibited trypsin molecules) ranged from 42 nM to 42 μM. Agar gels were used that were 2 mm thick and contained wells of 4 mm in diameter (volume about 25 μl). The agar contained a standard trypsin concentration of 42 nM. After diffusion the agar plates were stained as described in the Methods and the diameters of the uncolored, inhibited zones were measured. Figure 1A shows the increasing zone diameters in relation to higher Soybean Trypsin Inhibitor (STI) concentrations and prolonged diffusion periods for STI. The measured zone diameters are shown graphically in the graph of Figure 1B. A semilog plot

was found to approximate a straight line in the higher, but not in the lower concentration range. The curvature and the slope of the line depended both on the type of SPI (Figure 2) and the time of diffusion (Figure 1B). After overnight diffusion the differences in zone diameter between different SPI concentrations were much larger. Significant loss of sensitivity occurred after 48 hours of diffusion. Larger differences in zone diameter increase the accuracy of the determined SPI concentration and therefore overnight diffusion was adopted as the standard procedure.

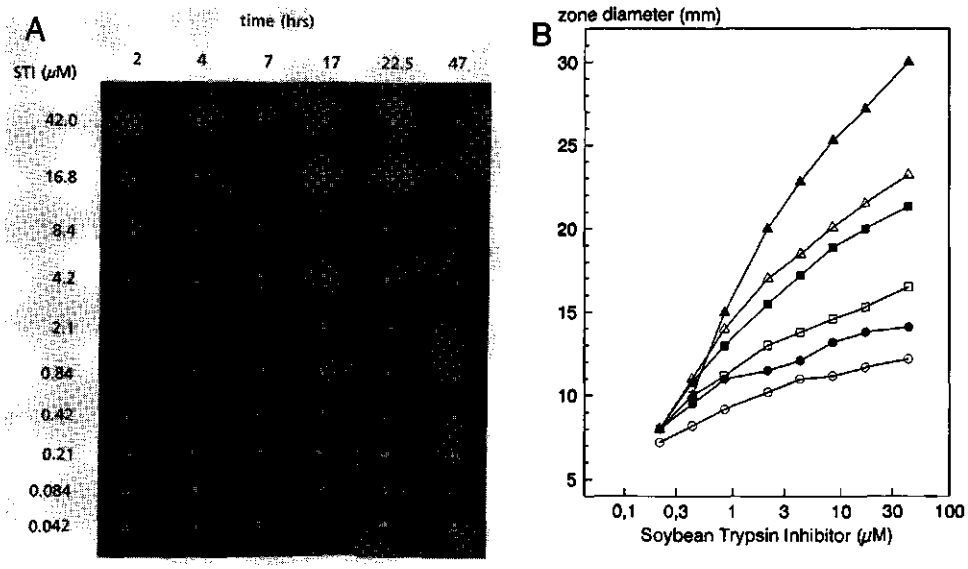

Figure 1. Effect of time and STI concentration on the diameter of the radial zones. Panel A shows the size of the inhibited zones after 2 to 47 hours of diffusion for STI concentrations ranging from 42 nM to 42 μM in agar plates containing 42 nM trypsin. B: A semilog plot of the zones in A showing the diameters of the zones after diffusion periods of 2 (○), 4 (●), 7 (□), 17 (■), 22.5 (△), and 47 (▲) hours.

Effect of the type of SPI on the speed of migration

The speed of migration of SPI molecules through the agar is driven by diffusion and depends to an important degree on the molecular weight of the SPIs involved. At identical SPI concentrations and diffusion periods large differences in the size of the inhibited zones of the five SPIs were found. Figure 2 shows the curves for all five SPIs after a diffusion period of 17 hours. The ranking order in terms of decreasing speed of migration at the highest PI concentration is: SBBI, STI, PI-2, α1-AT/OVOI. This order follows the ranking in terms of increasing molecular weight of the SPI multimers: 16, 20, 25, 51 /49 kD. This confirms the important influence of the molecular weight of the SPI on the speed of migration through the agar. From the way this order changes at low SPI concentrations especially with respect to α1-antitrypsin (α1-AT) (Figure 2) it is clear that other factors like the dissociation constant of the proteinase-SPI complex also have a large influence on the final size of the zone.

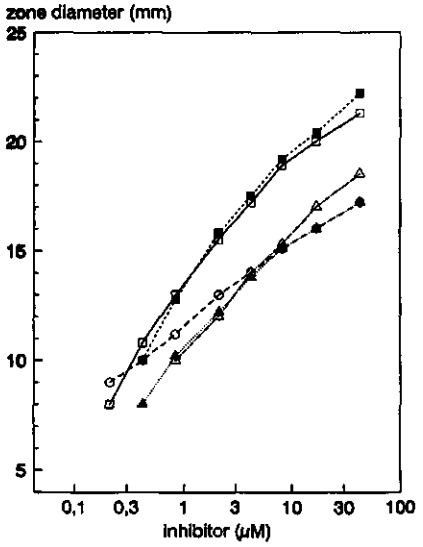

Figure 2. Effect of the type of inhibitor on the speed of migration. The semilog plot shows the variation in migrated distance between SBBI (■), STI (□), PI-2 (▵), OVOI (▲), and α1-AT (○) after 17 hours of diffusion in an agar plate containing 42 nM trypsin.

In order to quantify unknown, often mixed populations of SPIs in samples using this method one must make a specific reference line pertaining to a similar series of samples. First, the total SPI concentration in a sample with high activity is determined using a spectrophotometric assay as described in Methods. Next, the zone diameters of a serial dilution are measured and a reference line on a semilog plot is drawn. From this reference line, which is a smooth almost linear curve, SPI concentrations of samples that are known to differ only in the concentration of these SPIs can be determined directly from the size of an inhibited zone. The need to prepare a specific reference line for a certain SPI mix present in a series of samples makes the method best suited for larger numbers of samples or for frequently repeated measurements.

Factors influencing the sensitivity of the assay

The standard conditions that were employed (42 nM proteinase, 2 mm gel thickness, 4 mm well diameter, 16-18 hours diffusion) resulted in a sensitivity of detection of 2-20 pmol depending on the SPI measured. A low dissociation constant of the SPI-proteinase complex is likely to positively influence the sensitivity. This is confirmed by the observation that α1-AT, which is a very strong inhibitor of trypsin (< 1nM), displayed the highest sensitivity. After a short diffusion period of several hours α1-AT could be detected at a twofold higher concentration compared to the proteinase in the agar. Overnight diffusion resulted in disappearance of the inhibited zone at 84 nM α1-AT, most likely due to diffusion of unbound trypsin into the inhibited zone (Figure 1A). Unless the highest sensitivity is required overnight diffusion is preferred as it allows for more accurate SPI determinations on the larger size differences of the inhibited zones.

From our data with α1-AT it was obvious that the lower detection limit in the assay is determined to an important extent by the proteinase concentration in the agar. All of the proteinases tested except trypsin and pronase E showed a rapid staining of the agar at the

standard concentration. This allowed for further dilution of the proteinases to improve the sensitivity of the assay. For ovoinhibitor from chicken (OVOI) it was tested whether a 10-fold decrease in the subtilisin concentration in the agar was accompanied by a 10-fold increase in the sensitivity of detection. Figure 3A confirms that at the lowest proteinase concentration the zones are larger and can be easily measured. In addition the lowest OVOI concentration which was not detectable with the standard subtilisin concentration is clearly visible with diluted subtilisin in the agar. The only difference is that the boundaries of the zones are slightly more diffuse at low subtilisin concentrations. Figure 3B shows quantitatively that, indeed, dilution of the proteinase results in an increase of the sensitivity that approaches the dilution factor. At the tenfold diluted subtilisin concentrations the zone diameters for tenfold diluted OVOI samples tend to be slightly smaller. This can be explained by the fact that the concentration approaches the dissociation constant of the SPI-proteinase complex. In conclusion, for maximal sensitivity it is possible to use proteinase K, chymotrypsin and subtilisin at concentrations of 4 nM.

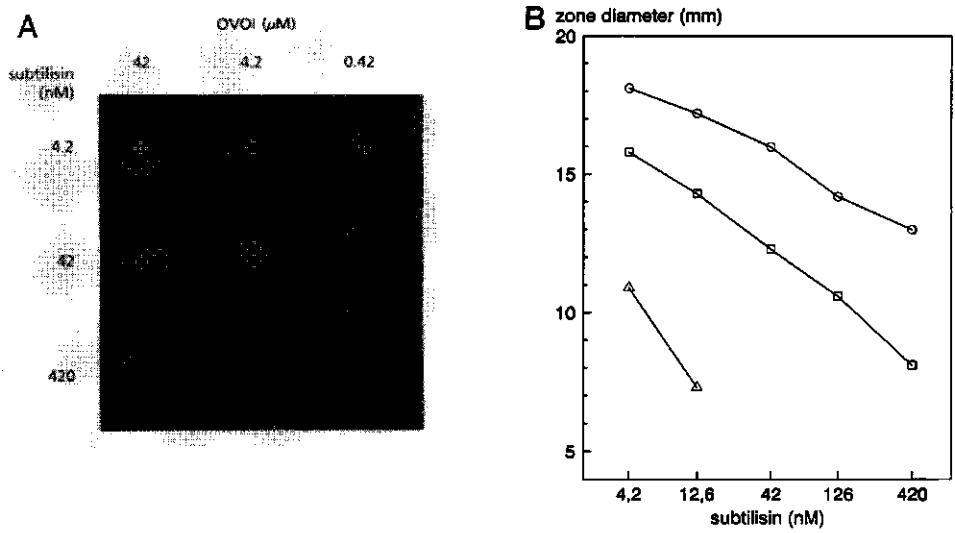

Figure 3. Increase of the sensitivity by reduction of the enzyme concentration. Panel A shows the size of the inhibited zones for dilutions of an OVOI containing sample on agar plates containing subtilisin in several dilutions. The semilog plot in panel B shows how the zone diameter decrease with increasing enzyme concentrations for samples containing 42 μM (○), 4.2 μM (□), and 0.42 μM (△) OVOI.

The sensitivity of the assay also depends on the quantity of the applied sample. This quantity is dependent on the dimensions of the well. Reducing the well diameter also reduces the zone diameter, and is, therefore, not very effective. Reducing the well depth, under ideal circumstances, should give the same zone diameter, while at the same time reducing the SPI sample volume needed to fill the well. It should, therefore, be a suitable second method to increase sensitivity if needed. This was investigated in the next section.

Effect of well depth on the zone diameter

The effect of the well depth was investigated by preparing plates with agar containing trypsin with a thickness of 1, 2 and 3 mm. A serial dilution of STI was allowed to diffuse for 17 hours. The final diffusion diameter was found to be slightly larger for shallow wells (Table 1). This difference is not expected to occur if the fluid in the well is not absorbed by the agar. However, under our experimental conditions the agar does absorb all the fluid within a few hours. This causes the bottom sides of the well to be exposed to the SPI solution for a longer time period. The color reaction only stains the top layer of about 1 mm, and, therefore, deeper wells produce slightly smaller inhibited zones. A well depth of 2 mm is preferred for the standard protocol as it is easier to prepare such plates, and because it reduces the pipeting error.

Table 1. Effect of agar gel thickness on the zone diameter

STI (μM)	Zone diameter (mm)		
	1.0 mm	2.0 mm	3.0 mm agar thickness
0.42	10.2	9.1	9.0
0.84	12.8	12.0	11.3
2.1	15.1	14.3	14.0
4.2	16.8	16.1	16.0
8.4	18.1	17.3	17.3
16.8	19.4	18.2	18.2
42.0	20.3	19.2	19.4

Note. Zone diameters were determined on 42 nM trypsin plates after 17 hours of diffusion

The accuracy of the SPI determination

The error in the determination of the SPI concentration of a sample is determined by a variety of factors. Inaccuracies in filling the wells exactly level to the height of the agar, imperfectly shaped wells or variation in the agar thickness and composition, lagperiods during the loading of the agarplate and errors in measuring the zone diameter all influence the final determined SPI concentration. To quantify these errors, wells on the same plate were filled four times with identical serial dilutions of STI and PI-2, which produced clear and vague outlined zones respectively. Radial inhibited zones were measured after 17 hours of diffusion. Table 2 shows that the standard deviation of the zone diameters varied from 0 to 0.3 mm for both STI and PI-2. Apparently, the vague boundaries of PI-2 inhibited zones can be determined as accurately as the clearcut boundaries of STI zones. From the slope of the semilog plot at 17 hours diffusion it was calculated that every 0.1 mm of error in the zone diameter equals 4% error in the SPI concentration value. Consequently, errors between subsequent measurements in one assay after 17 hours of diffusion will range from 4-12%. Inter-assay errors will mainly depend on variations in the enzyme concentration. The spectrophotometric determination of the enzyme concentration is more accurate than the quoted errors for the determination of the zone diameter and these intra-assay errors will therefore dominate the result.

Table 2. Accuracy in the determination of the zone diameter

Concentration (μM)	Zone diameter (mm)	
	STI[a]	PI-2
0.34	7.0 ± 0.0	-
0.67	10.1 ± 0.0	-
1.3	12.6 ± 0.1	8.0 ± 0.0
2.7	14.6 ± 0.3	10.6 ± 0.1
5.4	16.1 ± 0.0	12.3 ± 0.2
10.8	17.3 ± 0.1	14.0 ± 0.1
21.5	18.7 ± 0.3	15.6 ± 0.2
43.0	20.0 ± 0.0	17.3 ± 0.2

Note. Mean values (n = 4) ± standard deviation are given. Zone diameters were determined on 42 nM trypsin plates after 17 hours of diffusion

[a] This STI preparation was different from the preparation in table 1

Acetyl-phenylalanine-ß-naphtylester is a substrate for many serine proteinases

The usefulness of the described method would gain significantly if it were applicable to any serine proteinase of choice. A number of other serine proteinases were tested for staining of the agar via the diazo coupling of the ß-naphtol produced by the enzymatic hydrolysis of APNE. The five tested proteinases from mammalian, bacterial and fungal origin were all found to stain the agar purple in the following increasing order of strength: trypsin/pronase E < proteinase K/chymotrypsin/subtilisin. We expect that APNE can be used for detecting inhibitors of several other serine proteinases as well, establishing the broad application of the method.

It was tested whether the weaker substrate specificity of trypsin and pronase E could be compensated by using Nα-benzoyl-D,L-arginine-ß-naphtylamide (BANA) as a substrate (Johnston *et al.*, 1991). It was found that the rate of hydrolysis of BANA was at least 10-100 fold lower compared to the enzymatic hydrolysis of APNE for all five tested proteinases. Apparently, serine proteinases perform esterolytic hydrolysis at a much higher rate than amidolytic hydrolysis. It will be worthwhile to synthesize and test APNE-like substrates substituted with other amino acids. More specific or better substrates may thus be found. With the availability of other APNE-like substrates it may also be feasible to apply the described method to cysteine proteinases. The spectrophotometric protocol by Barret (1972) for the detection of cysteine proteinase inhibitor activity was modified to fit the described assay system. It was found that the method could work for cysteine proteinases as well, but that it was 100-fold less sensitive using both BANA and APNE as substrates for papain due to insufficient staining.

DISCUSSION

A radial SPI diffusion assay is described which improves the method as published by Kourteva *et al.* (1987) on a number of counts. The accuracy was improved as a volume ten times larger (25 μl) is pipetted into a well of defined diameter instead of placing a 2 μl droplet on top of an agar layer. The high sensitivity of up to 0.1 pmol STI was maintained

as it was shown that a tenfold decrease in the proteinase concentration and a decrease in the depth of the well can compensate for the loss of sensitivity due to the larger volume. The sensitivity of the method matches the sensitivity of other spectrophotometric (Melrose and Ghosh, 1992), diffusion (Gallagher *et al.*, 1986; Shukle and Murdock, 1983), or immunological methods (Ryan, 1967). It was shown how prolonging the diffusion period from several minutes to hours or days can increase the range of quantifiable SPI concentrations. The assay is applicable to a wider range of serine proteinases also from fungal and bacterial origin and the molecular weight of the SPIs involved has an important influence on the speed of migration through the agar. Finally, the assay procedure was improved with respect to the quantitative presentation of the results. Unknown SPI activities can be measured in terms of their molar concentration of SPI active sites which was not described in the original method.

We have found the method to be particularly useful for following SPI induction in plants in response to different forms of stress (Jongsma *et al.*, 1994). The method allowed us to analyze hundreds of samples for their SPI levels in a short time period. In addition we could use purified proteinases from fungal and bacterial origin to study whether a pathogen attack on the plant induced an SPI response specific for these proteinases from pathogenic origin.

CHAPTER 3

TRYPSIN INHIBITOR ACTIVITY IN MATURE TOBACCO AND TOMATO PLANTS IS MAINLY INDUCED LOCALLY IN RESPONSE TO INSECT ATTACK, WOUNDING AND VIRUS INFECTION

Wounding of plants by insects is often mimicked in the laboratory by mechanical means like cutting or crushing, and has not been compared directly to other forms of biotic stress like virus infection. To compare the response of plants to these types of biotic and abiotic stress, trypsin inhibitor (TI) activity induced locally and systemically in mature tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* L.) plants was followed for 12 days. In tobacco, cutting, crushing and insect feeding all induced comparable levels of TI activity of approximately 5 nmol per mg leaf protein in wounded leaves, while TMV infection of tobacco induced 10-fold lower amounts in the infected leaves. In tomato, feeding by insects led to the induction of a level of TI activity of also 5 nmol per mg leaf protein. In contrast, both cutting and crushing of tomato leaves induced 10-fold higher amounts. These data show that biotic stress in the form of insect feeding and TMV infection or abiotic stress in the form of wounding have different effects on local levels of induced TI activity in mature tobacco or tomato plants. Irrespective of the type of wounding in neither tobacco nor tomato, systemic induction of TI activity could be observed in nearby unwounded leaves, which suggests that systemic induction of TI activity in mature tobacco and tomato plants is different compared to systemic TI induction in seedlings. Wounding of tobacco leaves, however, did increase the responsiveness to wounding elsewhere in the plant as measured by an increased induction of TI activity.

INTRODUCTION

Green and Ryan (1972) were the first to report the induction of proteinase inhibitors (PI's) in potato and tomato leaves in response to leaf feeding by adult Colorado beetles. This suggested a role for PI's in plant defense against insects. PI's could also be induced by mechanical wounding and results of such experiments are generally extrapolated to a plant's response to defoliating insects (Ryan 1978, 1992). However, preliminary data have been reported on the induction of PI's by insect feeding which suggest that this is not the case for mature tomato plants. Duffey and Felton (1989) showed that the induction of PI activity by feeding *Heliothis zea* larvae may be much less than by mechanical wounding. In addition, systemic induction of PI activity after mechanical wounding was only detected in unwounded leaves of very young tomato plants, but not in unwounded leaves of mature tomato plants (Wolfson and Murdock 1990).

Nevertheless, expression of PI genes in transgenic tobacco plants has been shown a strategy for enhancing crop resistance to insect pests (Hilder *et al.* 1987; Johnson *et al.* 1989;

Hoffmann *et al.* 1992). This may be the result of adaptation of insects to PI's of their host plant species, causing heterologous PI's to be more effective. On the other hand, the level of transgenic PI activity needed to induce insect resistance in plants may be a function of the level of induced endogenous PI activity. If endogenous PI activity is induced to high levels after wounding by insects a lower amount of transgenic PI activity might be sufficient to induce insect resistance.

In this context we determined the local and systemic induction of endogenous trypsin inhibitor (TI) activity in mature tobacco and tomato plants after wounding by feeding insects (*Manduca sexta*). We compared this induction to the local and systemic induction of TI activity during daily repeated mechanical wounding by cutting or crushing or after TMV infection to see if insect feeding may be mimicked by mechanical wounding or viral infection of these plant species.

MATERIALS AND METHODS

Plants

Seeds of *Nicotiana tabacum* L. cv Samsun NN (obtained from Dr. V.A. Hilder, Dept. of Biological Sciences, Univ. of Durham, Durham, UK), and *Lycopersicon esculentum* Mill. cv Moneymaker (CPRO-DLO, no. 87150) were sown in pot soil and seedlings were transferred to small plastic pots 7-10 days upon sowing. After three weeks plants were transferred to square plastic pots of 5 liter volume. This sustained vigorous growth of the plants until the end of the experiments. Until the start of the experiment plants were grown in the greenhouse with supplementary high pressure sodium light providing a minimum light intensity (excluding daylight) of on average 10 Wm^{-2} for tobacco and 17 Wm^{-2} for tomato under a temperature regime (21/18 ^{0}C) following a 16/8 h day/night rhythm. Experiments were done 3 times, starting at 19th October, 9th and 24th November 1992. Tomato plants were 42 days old and tobacco plants 49 days at the start of the experiments. At this stage tomato had 7 expanded leaves above the hypocotyls and tobacco had 7 leaves larger than the radius of the pot. Twelve days later, at the end of the experiment, plants had ca. 15 expanded leaves. The temperature in the greenhouse during the experiments was kept at a constant temperature of $25^{0}C$ and a relative humidity (RH) of 80% during the day and 70% during the night under the same conditions of supplemental light.

Upon transfer to climate controlled growth chambers, plants were given 3 days to adjust to the new conditions: 40 Wm^{-2} fluorescent light during a 16 h day and 70% RH. Temperatures were kept constant at 25 or 32 ^{0}C.

Plant treatments and sampling

Groups of 36 tomato and tobacco plants were given the following treatments: none (control), feeding by *M. sexta* larvae, cutting, crushing, TMV infection (tobacco only), mock infection (tobacco only). Each day at noon plants were wounded (cutting and crushing) and samples were collected from leaves 3, 5, and 7 of three plants drawn from each of the groups in order to obtain data on both local and systemic effects at that day. The leaf tips of the untreated leaves 3 and 7 were sampled after first removing the midrib. For tomato leaf number 1 was the first emerging leaf above the hypocotyls. For tobacco leaf number 1 was the first leaf larger than the radius of the pot (ca. 9 cm). Sampled leaf pieces were pooled.

Once plants had been sampled they were discarded.

Larvae

Leaf 5 of tobacco and tomato plants was hulled in microperforated cellophane bags closed around the petiole with staples to prevent larvae from escaping. Three newly hatched first instar *Manduca sexta* (Lepidoptera: Sphingidae) larvae (Carolina Biological Supply, Burlington, NC, USA) were placed on top of the leaf through a cut in the bag. With tomato the insects were placed on the outermost three leaflets. The larvae were left to feed inside the bag for twelve days. Leaf samples were taken there where the larvae had most recently been eating. Larvae were collected and weighed. Leaf damage was recorded by hand using a schematic drawing of a leaf. Damage was estimated using an image analyzer.

Cutting

Cutting was done every day with a pair of scissors, removing a 1-2 cm wide section across the midrib of leaf 5 of tobacco. In case of tomato a leaf strip of 2-3 mm was removed from all 7 major leaflets of leaf 5. The wounding treatments were such that at day 12 approximately one quarter of the leaf or leaflet remained for sampling. Leaf samples of about 2x3 cm were taken immediately next to the cut near to the leaf edge.

Crushing

Leaves of tobacco were crushed every day by strongly pressing it with flat tweezers across the midrib of leaf 5 up to two centimeters from the leaf edge, creating a band of macerated tissue about 0.5 cm wide. Successive crushed bands were about 1-2 cm apart. Seven tomato leaflets were crushed in a similar way but no space was left between successive crushings. Leaf samples were taken next to the crushed leaf area near to the leaf edge on the leaf base side of the wound.

TMV

Leaf 5 of tobacco plants was powdered with carborundum powder and inoculated with 0.2 ml of a purified TMV preparation (3300× stock, obtained from Dr. H.J.M. Linthorst (Dept. of Biochemistry, Leiden University, Leiden, The Netherlands). Mock-infected carborundum controls were included to check for wound-induction by the carborundum treatment. The midrib and the leaf edge were avoided during the inoculation to allow sampling to continue until day 12. 100-200 necrotic lesions per leaf developed. Samples were taken from the leaf edge halfway the leaf immediately adjacent to the necrotic lesions.

Protein isolation

Three sampled leaf strips of three plants were pooled in an Eppendorf tube and immediately frozen in liquid nitrogen. Frozen leaf material was ground using a mechanically driven metal mortar cooled in liquid nitrogen. The ground leaf material was thawed on ice and mixed with 0.3 ml extraction buffer: 0.1 M Tris-Cl, pH 7.6; 5% polyvinylpolypyrrolidone (Sigma, St. Louis, MO, USA); 2 $mgml^{-1}$ phenylthiourea (Sigma); 5 $mgml^{-1}$ diethyldithiocarbamate

(Sigma), 0.05 M Na_2EDTA. The extraction buffer partially prevented phenolic oxidation of protein in those samples that had been subjected to wounding for many days. Samples were centrifuged immediately at maximum speed (12000 g) for 20 min at 4 °C, and 0.3 ml supernatant was transferred to a tube containing 1.2 ml ice-cold, saturated ammonium sulphate solution. Protein was left to precipitate for one hour on ice, was centrifuged for 10 min at maximum speed at 4 °C, and the supernatant was carefully removed. Protein pellets were redissolved in 0.2 ml (tobacco) or 0.4 ml (tomato) Tris buffer (0.1 M, pH 7.6). Insoluble material was removed by centrifugation (12000 g, 10 min, 4 °C). The protein concentration of the samples was then determined in duplicate by the method of Bradford (1976).

Radial diffusion assay

The radial diffusion assay used for the determination of the inhibitor activity against trypsin and subtilisin has been described previously (Jongsma *et al.* 1993). The only modification was the use of 20% (v/v) dimethylformamide in the staining solution. In brief, the method is based on the radial diffusion of a protein extract from a central well through an agar gel containing a serine proteinase at either 40 or 4 nM concentration. The active sites of trypsin and subtilisin were titrated as described to obtain the molar concentration of active enzyme (Chase and Shaw 1967; Bender *et al.* 1966). After 18 h incubation at 4°C the agar gel was stained via the diazo coupling of the β-naphtol produced by the enzymatic hydrolysis of N-acetyl-DL-phenylalanine-β-naphtylester. Circular zones containing inhibitor-proteinase complexes remained colorless, while the region containing only proteinase showed a bright pink-purple color. A reference curve related the diameter of the colorless zone to the logarithm of the proteinase inhibitor concentration. Levels of PI's are reported as nmol inhibited proteinase molecules per mg soluble leaf protein. The actual molar PI concentration may be lower in case of multiheaded PI's. Zone diameters of crude extracts were converted to PI activity by titrating trypsin and subtilisin (previously titrated) with these extracts using azocasein as a substrate. The slope of a reference curve depended to an important degree on the dissociation constant of the proteinase-PI complex and the molecular weight of the PI. Therefore, reference curves were made for every type of treatment in order to obtain values for PI activity independent of the set of PI's induced.

RESULTS

In tobacco, TI activity is mainly induced locally in response to mechanical wounding

To determine the influence of growth conditions on the induction of TI activity upon wounding, tobacco plants were grown in the greenhouse or growth chamber and at two temperatures. The plants were wounded for four subsequent days by either cutting or crushing. Subsequently, samples were taken immediately next to the wound, 10 cm removed from the wound at the leaf base, two leaves above, and two leaves below the wounded leaf. Fig. 1 shows the TI activity in the wounded leaves. More TI activity was measured in the wounded leaves of plants grown in the growth chamber compared to plants grown in the greenhouse at both 25°C as well as 32°C and irrespective of the type of wounding. Apparently factors other than temperature positively influenced TI induction in the growth

chamber. Ten cm away from the woundsite a significantly lower TI activity level of approx. 60% was induced. This was even more prominent in non-wounded leaves. Only a very limited systemic induction of TI activity could be measured in higher and lower leaves. No significant difference between induction of systemic TI activity by cutting or crushing could be shown.

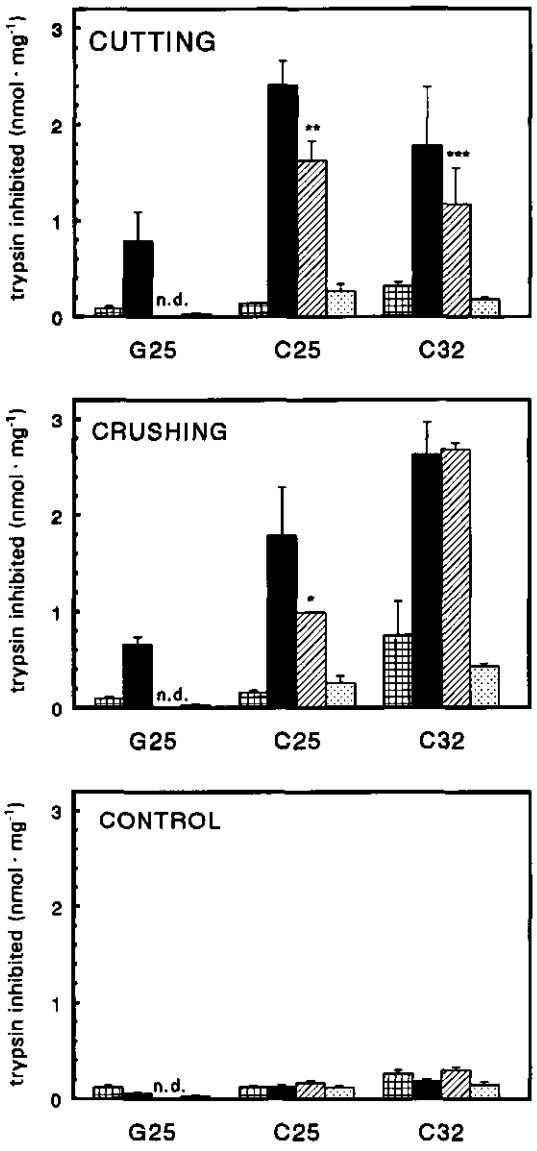

Figure 1. Influence of environmental conditions and types of wounding on the induction of TI activity in tobacco. The treatments are encoded by G, greenhouse conditions; C, climate controlled chamber conditions; 25 or 32, constant temperatures in degrees Celcius. TI activity was monitored by radial diffusion assay after 4 days of wounding leaf 5 daily by cutting or crushing. Activity is expressed as nmol inhibited trypsin molecules per milligram extracted protein. Standard error bars are given (n=4 in C; n=3 in G). TI activities were measured in lower leaf 3 (▦), wounded leaf 5, immediately next to the wound (■), leaf 5, 10 cm away from the wound at the leaf base (▨), higher leaf 7 (▩). Significance is given for activity levels 10 cm away from the wound relative to close to the wound: $^{*}p<0.05$; $^{**}p<0.02$; $^{***}p<0.01$ (Student's t-test on percentage transformed data to correct for individual plant differences).
n.d. is not determined

Induction in time of TI activity by Manduca sexta feeding and mechanical wounding

Next, the induction of TI activity in time in response to insect feeding and wounding was followed. Because development to pupation of lepidopteran larvae is in the order of weeks we followed the TI induction for twelve days. Fig. 2 shows the percentage leaf area removed from tobacco and tomato leaf 5 during this period. The three terminal leaflets of tomato leaf 5 were completely ingested at day 6. At this point larvae moved to the remaining four leaflets and leaf samples were taken there. Tobacco leaves were less rapidly ingested than tomato leaves. This was correlated with the lower weight gained by the larvae on tobacco, which was only 25% of the weight gained on tomato leaves.

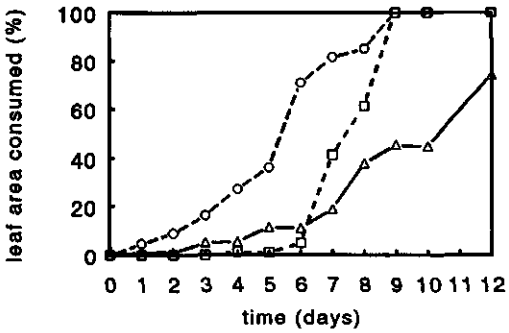

Figure 2. Percentage tobacco or tomato leaf area consumed by *M. sexta* larvae. Consumed leaf area of tomato leaf 5 is split up in two parts: (○—○) consumed leaf area of the three terminal leaflets of leaf 5 for which sampling continued until day 5; (□---□) consumed leaf area of the remaining four basal leaflets for which sampling started here on day 6. Leaf damage of tobacco leaf 5 (▵—▵) was distributed uniformly over the leaf.

Initially, young *Manduca sexta* larvae most potently induced TI activity in tobacco (Fig. 3A,C) and tomato (Fig. 3B,D). In tobacco the induced levels increased linearly until day 12 (Fig. 3A) up to a TI activity level of about 5 nmol/mg. In tomato this level was already reached at day 5 and remained constant then. Systemic induction of TI activity by newly hatched *M. sexta* larvae was not observed before day 10 of their development on neither tobacco (Fig. 3E,G), nor tomato (Fig. 3F,H), and was small compared to local levels in all cases.

The induction of TI activity in tobacco and tomato in response to daily repeated mechanical wounding is also shown in Fig. 3. In tobacco the highest TI activity level of 8 nmol/mg could be induced by cutting (Fig. 3A) while in tomato crushing leaf tissue induced even a level of 50 nmol/mg (Fig. 3B). Again, systemic induction of TI activity in both lower and higher leaves of tobacco and tomato was only minor and remained less than 5% of the level observed close to the wound site.

Systemic influence of wounding on the wound-inducibility of TI activity in leaves of tobacco

To determine the systemic influence of wounding a lower leaf on the wound-inducibility of TI activity in a higher leaf, the influence on TI activity induction of simultaneously wounding separate tobacco leaves is shown in Fig. 4. TI activity in leaf 5 and 7 were assayed after 6 days of treatment. Daily wounding of leaf 7 induced TI activity in the wounded leaf while systemic induction in the unwounded, lower leaf 5 was negligible. Wounding of leaf 5 induced a similar TI activity level in the wounded leaf while the systemic induction in leaf 7 was very low, but nevertheless significant. After simultaneous wounding of leaf 5 and 7, the TI activity level in the lower leaf 5 was not significanctly increased compared to

wounding leaf 5 only. However, the TI activity in leaf 7 was more than doubled compared to no wounding of the lower leaf. This suggests that though wounding does not systemically induce high levels of TI activity in non-wounded leaves of tobacco, there is a mechanism which influences the level of response to wounding of higher leaves.

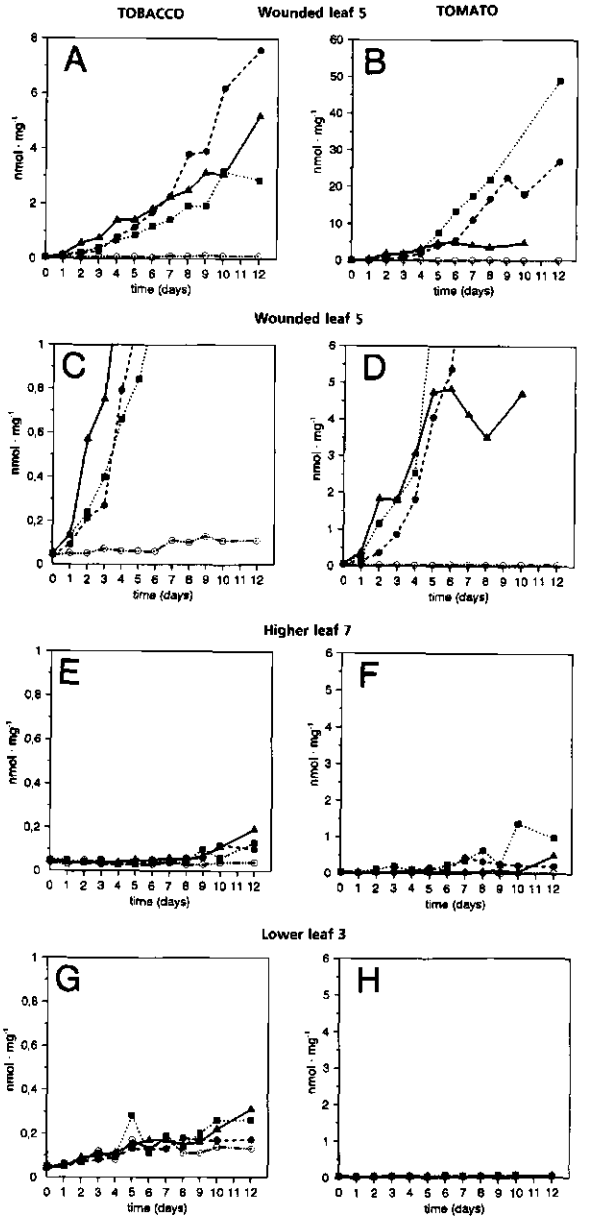

Figure 3. Time course of local and systemic induction of TI activity in tobacco and tomato in response to several forms of wounding. The left column of graphs shows TI induction in tobacco, the right column TI induction in tomato. Activity is expressed in terms of nmol inhibited trypsin molecules per milligram of extracted protein. **A,B:** Activity induced in the wounded leaf 5. **C,D:** The same graphs as A,B but on an 8 and 10 fold smaller scale. **E,F:** Activity induced in leaf 7 two leaves above the wounded leaf. **G,H:** Activity induced in leaf 3 two leaves below the wounded leaf. The lines denote the following treatments: (▲—▲) feeding by *M.sexta* larvae; (●—●) cutting; (■···■) crushing; (○-·-·-○) unwounded control. The trypsin inhibitor activity was monitored by radial diffusion assay. Every datapoint is based on nine plants from three independent experiments under greenhouse conditions.

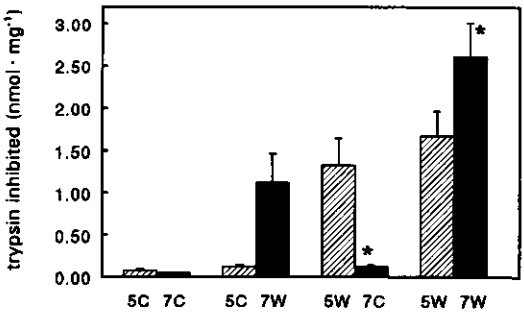

Figure 4. Systemic influence of wounded lower leaves on the inducibility of TI activity in higher leaves. The four sets of columns represent the TI activity induced in leaf 5 (▨) and leaf 7 (■) in four different treatments of six days: 5C 7C, both leaves unwounded; 5C 7W, only leaf 7 wounded by cutting; 5W 7C, only leaf 5 wounded by crushing; 5W 7W, both leaves wounded by crushing and cutting respectively. The trypsin inhibitor activity was monitored by radial diffusion assay. Every value is an average of nine plants from three independent experiments under greenhouse conditions. In each experiment samples from three plants were pooled. Standard error bars are given (n=3). Significance is given relative to the treatment with the opposite leaf unwounded: $^{*}p<0.05$ (Student's t-test).

Comparison of the induction of TI and subtilisin inhibitor (SI) activity by TMV infection and M. sexta larvae

It has been shown that in tobacco TMV infection specifically induced Proteinase Inhibitor I (PI-1) with activity specific for subtilisin and only a negligible level of TI activity (Geoffroy *et al.* 1990). To determine the specificity classes of PI's induced by *M. sexta* in tobacco and to compare the strength of their induction, TMV infections of tobacco were carried out under identical environmental conditions and simultaneous with the insect-wounding experiments. The induction of TI activity in tobacco during the hypersensitive response to TMV was about 10 times lower in comparison to the induction in response to insects (Fig. 5). TI activity was first observed at day 4 at the onset of the formation of necrotic lesions which is three days

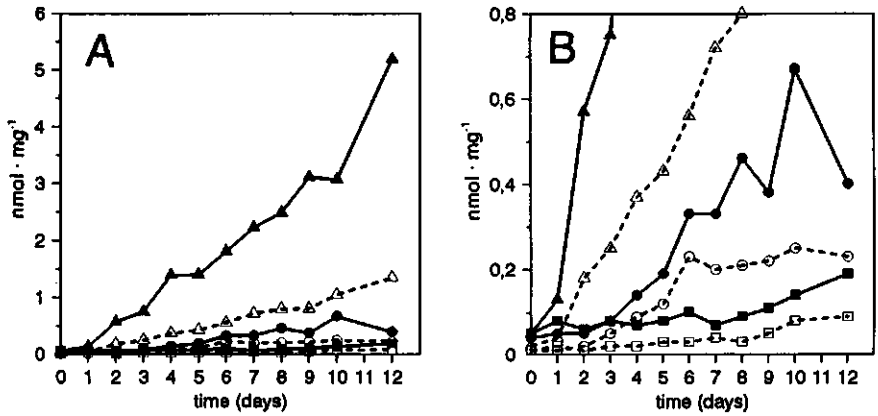

Figure 5. Comparison of the trypsin and subtilisine inhibitor activity induced by insects and TMV. Panel **A** is identical to panel **B** but drawn on a larger scale. Trypsin activity is marked by solid lines, subtilisin activity by dashed lines. *Trypsin inhibitor activity* induced by *M. sexta* larvae (▲—▲), TMV (●—●) and carborundum control (■—■). Subtilisin inhibitor activity induced by *M. sexta* larvae (△---△), TMV (○---○) and carborundum control (□---□).

later than the response to feeding insects. Fig. 5 also shows the induction of SI activity by insects and TMV infection. The level of SI activity induced by insects was about 30% relative to the induced level of TI activity, while after TMV infection the level of SI and TI activity was equal up to day 6. After day 6 the relative SI activity decreased to on average 60% of the level of TI activity.

PI's of different native molecular weight may be distinguished by comparison of the reference lines obtained in the radial diffusion assay (Jongsma *et al.* 1993). Fig. 6 shows the reference curves determined for TI and SI activity induced by wounding (cutting, crushing or insects) and TMV. The reference curves for TI activity were virtually identical, which suggested the induction of a PI population of similar native molecular weight by both TMV and insects. The slope of the SI reference curve for wounding was identical to the slope of the TI reference curves. The shift can be explained by the use of plates containing 4 nM instead of 40 nM proteinase concentrations. Agar plates containing 40 nM subtilisin gave a reference curve for wounding almost identical to the TI reference curves for wounding and TMV (data not shown). This also provides an indication that the SI activity may be contributed by (a) subtilisin inhibitor(s) of molecular weight similar to the trypsin inhibitor(s). The slope of the SI activity reference curve after TMV infection was not as steep, suggesting the presence of a higher molecular weight PI. These data suggest that TMV infection induces a type of SI, which is not highly induced by insect damage.

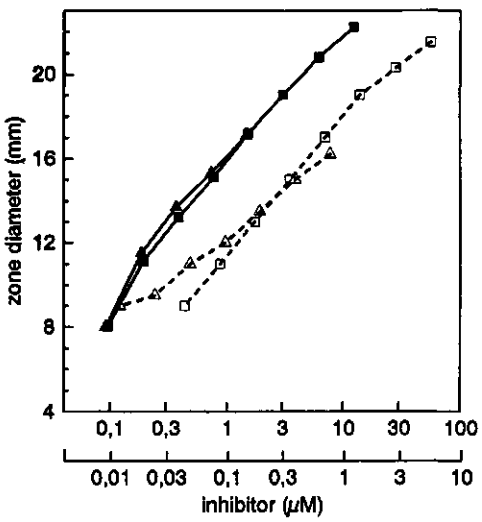

Figure 6. Comparison of reference lines of TMV and wound-induced PI activity in tobacco. Reference lines relating the zone diameter to the logarithm of the PI concentration are shown. Reference lines for trypsin are given by solid lines. Zone diameters were determined after 18 hr diffusion on 40 nM trypsin agar plates. The top x-axis scale is applicable. Reference lines for subtilisin are given by dashed lines. Agar plates contained 4 nM subtilisin and the lower x-axis scale is applicable. TI activity induced by wounding (■—■), and TMV (▲—▲). SI activity induced by wounding (□--□) and TMV (○--○).

DISCUSSION

Induction of TI activity by M. sexta and mechanical wounding

In both tobacco and tomato leaves TI activity was strongly induced near the wound site in response to feeding by *M. sexta* larvae. In tobacco TI activity increased linearly to a final level of 5 nmol/mg at day 12 at which point the leaf was nearly fully ingested. In tomato the induction was more rapid reaching a similar level at day 5 while remaining constant

afterwards (Fig. 3D). At day 5 the original outer 3 leaflets on which the larvae were placed were nearly completely ingested. Subsequently, the larvae moved to fresh inner leaflets of leaf 5 of tomato and sampling continued there. The constant level observed in those newly attacked leaflets did not represent a physiological limit to the level of TI activity, because the levels induced by mechanically wounding the same leaflet for the entire period were 5-10 fold higher. Therefore, the constant level observed in three independent experiments, indicates that unwounded leaflets on a tomato leaf attacked by insects were not systemically induced to levels found in the wounded leaflets or that after day 5 the larvae were eating the leaves too fast for the plant to respond with maximum TI activity levels.

During the first 4-5 days insect feeding showed a stronger induction of TI activity in the wounded leaf than daily repeated mechanical wounding. After the initial period cutting in tobacco and crushing and cutting in tomato resulted in higher induced TI activity levels. These high levels were induced especially after the first 4-5 days of wounding, as if some mechanism amplified the wound response at that point.

As judged by the radial diffusion reference lines our data suggest that mechanical wounding induces the same set of PI's in all three wounding treatments (data not shown). However caution should be exercised in extrapolating results based on mechanical wounding to feeding insects. Feeding insects damage plants differently from mechanical wounding in terms of frequency, distribution and form. These and other factors like the rate of leaf consumption result in patterns of PI activity induction that can deviate strongly from mechanical wounding.

Systemic TI activity induction

Regardless whether leaves were ingested by insects for twelve days or mechanically wounded by cutting or crushing every day, our data indicate that systemic induction of TI activity in nearby unwounded leaves is negligible in mature tobacco and tomato plants. This is in contrast to reports on strong and rapid systemic wound-induced accumulation of PI's in tomato and tobacco by crushing (Nelson *et al.* 1983; Wildon *et al.* 1992; Pearce *et al.* 1993) or feeding insects (Green and Ryan 1972; Broadway *et al.* 1986). However, these experiments were always carried out with expanding new leaves of very young plants, never older than 3 weeks. Wolfson and Murdock (1990) reported that systemic induction of TI activity in response to mechanically wounding tomato disappeared with aging/size, becoming negligible in 35 days old plants. The absence of systemic TI activity in tomato has been confirmed and extended to tobacco and to damage by insects. However, by comparison of local and systemic TI levels it is shown at the enzymatic level that the lack of systemic induction is not accompanied by a loss of TI induction close to the wound. Analogously, it was shown by Northern blots (Linthorst *et al.* 1993) that in mature tobacco a subtilisin-specific PI-1 gene could only be induced locally and not systemically by wounding. In mature plants locally induced PI's should, therefore, be expected to also contribute to the level of insect resistance observed in mature plants (Wolfson and Murdock 1990).

In tobacco we measured the TI activity level both next to the wound and about 10 cm away from the wound on the same leaf. Across this short distance a significant drop in TI activity of 35-45% was observed. The TI activity level systemically induced in non-wounded leaves is very low compared to the level induced close to the wound-site. This gradual reduction suggests that dilution (chemical) or damping (electrical) of wound signals (Farmer and Ryan 1990, Pearce *et al.* 1991; Wildon *et al.* 1992) may diminish their effectiveness.

The small systemic response in older plants compared to the large response in very young plants may thus be understood as that a larger biomass can reduce the signal strength. Alternatively, the pattern of systemic induction by a phloem-translocated wound-signal like the small polypeptide systemin (Pearce *et al.* 1991) may be under control of source-sink relationships between older and younger leaves. During the experiments higher leaves that were sampled functioned as sink-leaves for only a few days due to rapid outgrowth of the plants and this may have suppressed PI induction in those leaves. On the other hand, it may also be that the induction of PI activity is under a form of negative control like the level of auxin (Kernan and Thornburg, 1989; Thornburg and Li, 1991). Our data suggest that simultaneous wounding of a lower leaf more than doubled the response to wounding in a higher leaf, but without wounding the higher leaf hardly any PI activity was induced. Apparently, the higher leaf received information that doubled the response to a trigger.

Both TMV and insects induce a higher level of trypsin activity than subtilisin activity in tobacco

Upon TMV infection a relative level of TI activity almost twice as high as the level of SI activity was found in tobacco leaf material immediately adjacent to necrotic lesions formed. Upon wounding TI activity is more than 3 times as high as SI activity. TMV infection, therefore, did not selectively induce inhibitor activity of microbial serine proteinases. Geoffroy *et al.* (1990) showed that TMV induced SI activity approximately 20 times stronger than TI activity. While we used the same tobacco cultivar and the same source of enzymes this discrepancy might be explained by the different biochemical methods used or by differences in experimental conditions.

As indicated by the speed of migration through the agar all wound-induced TI and SI activity behaved the same. However, SI activity induced by TMV infection migrated more slowly. This suggests the induction by TMV infection of a PI of larger native molecular weight. This might be Proteinase Inhibitor I (PI-1) as purified by Geoffroy *et al.* (1990). Linthorst *et al.* (1993) showed that PI-1 mRNA is also induced in a mechanically wounded leaf, however, 2-3 times weaker than upon TMV infection. Our data showed that feeding insects induced SI activity three times stronger compared to TMV infection which confirmed the conclusion of Heitz *et al.* (1993) that the contribution of this PI-1 inhibitor to SI activity induced by abiotic stress is minor.

Recently, the wound-inducible proteinase inhibitors from tobacco were characterized as truncated members of the PI-2 family (Pearce *et al.* 1993; Atkinson *et al.* 1993). Their activity towards subtilisin was not investigated, but members of the PI-2 family possess activity towards subtilisin as well (unpublished observation). This is in agreement with our finding that wounding appeared to induce inhibitors with an 'average' native molecular weight similar to PI-2.

Consequences of endogenous PI activity for PI-based insect resistance in transgenic tobacco plants

Insect resistance in transgenic tobacco plants based on expression of a heterologous proteinse inhibitor gene was first reported for the cowpea trypsin inhibitor (CpTI) (Hilder *et al.* 1987). These tobacco plants were reported to contain 1% CpTI per total soluble leaf protein. CpTI is a double headed trypsin inhibitor of 8 kDa, so that the predicted molar TI concentration

is 2.5 nmol/mg. Transgenic tobacco plants resistant to *M. sexta* larvae expressing potato inhibitor II (PI-2) from potato were reported to contain 332 μg PI-2 per gram tobacco leaf (Johnson *et al.* 1989). The average concentration of soluble protein we measured in tobacco leaves was 10 mg/g and the molecular weight of PI-2 is 12 kDa. Therefore, the predicted molar TI concentration was about 2.8 nmol/mg in these plants. In tobacco such PI levels were induced by *M. sexta* larvae by day 7 and in tomato by day 4. While the reported resistance of transgenic plants was caused by the transgenic PI, the induction of endogenous PI's ranging multiple specificities during insect feeding as described in this paper might have positively influenced the level of resistance. In the same line of reason, the strong influence of environmental conditions on PI induction might affect the resistance level of transgenic plants and it might be speculated that the level of insect resistance upon transfer of PI genes to other plant species might be negatively affected by absence of endogenously induced PI's.

CHAPTER 4

ADAPTATION OF *SPODOPTERA EXIGUA* LARVAE TO PLANT PROTEINASE INHIBITORS BY INDUCTION OF PROTEINASE ACTIVITY INSENSITIVE TO INHIBITION

Tobacco plants were transformed with a chymotrypsin/trypsin-specific proteinase inhibitor II (PI2) cDNA clone of potato under the control of a constitutive promoter. Although considerable levels of transgene expression could be demonstrated, the growth of larval *Spodoptera exigua* fed with detached leaves of PI2-expressing plants for six days was not affected. Analysis of the composition of tryptic gut activity demonstrated that only 18% of the proteinase activity of insects reared on these transgenic plants was sensitive to inhibition by PI2, whereas 78% was sensitive in insects reared on control plants. Larvae had compensated for this loss of tryptic activity by a 2.5-fold induction of new activity insensitive to inhibition by PI2. PI2-insensitive proteolytic activity was also induced in response to endogenous proteinase inhibitors of tobacco, demonstrating that induction of such proteinase activity represents a natural mechanism via which plant-feeding insects overcome plant proteinase inhibitor defense.

INTRODUCTION

The involvement of proteinaceous proteinase inhibitors (PIs) in plant defense against leaf feeding insects has been recognized since the earliest discovery that they were induced in response to insect attack (Green and Ryan, 1972; Jongsma *et al.*, 1994). Experiments with artificial diets and a wide range of insects confirmed the antinutritional effects of proteinaceous PIs (Broadway *et al.*, 1986; Burgess *et al.*, 1991, 1994; Johnston *et al.*, 1993; Orr *et al.*, 1994; Steffens *et al.*, 1978) although some negative results were also reported (Purcell *et al.*, 1992). The expression of heterologous proteinase inhibitors in transgenic tobacco plants provided final confirmation *in planta* for their roles as resistance factors although protection was only partial (Hilder *et al.*, 1987; Johnson *et al.*, 1989).

Recently, the effectiveness of proteinase inhibitors was suggested to depend on the affinity or specificity of an inhibitor for the main gut proteinases of an insect (Burgess *et al.*, 1991; Gatehouse *et al.*, 1993; McManus *et al.*, 1994), but the mechanism of action and effect of proteinase inhibitors is only partially understood. Broadway and Duffey (1986) showed that gut proteinase activities of *S. exigua* and *Heliothis zea* were similar or increased when larvae were chronically exposed to high levels of PI2 or soybean trypsin inhibitor in artificial diets. The simple scenario that growth rates were reduced due to reduced rates of proteolysis (Reese, 1983) was, therefore, dismissed. Instead, these results were interpreted by Broadway and Duffey (1986) to suggest that a feedback mechanism was leading to the hyperproduction of proteinases to compensate for the loss of activity, which in turn led to the depletion of essential amino acids, and finally resulted in retarded growth rates.

Our aim was to establish to what extent and how PI2 expressed in tobacco leaves would affect larval growth and digestive physiology of *S. exigua*. Our data show that *S. exigua* larvae adapt to PIs by induction of gut proteinase activity insensitive to inhibition.

MATERIALS AND METHODS

Vector construction and plant transformation

The binary plant vector pCPO33 was derived from pCPO31 (Van der Salm *et al.*, 1994) by removing the *Hin*dIII fragment containing the TR 2'/1' promoter cassettes. Subsequently, the *Nco*I-*Hin*dIII fragment containing the 35S promotor was replaced by the *Nco*I-*Eco*RI fragment containing the double 35S promotor with AlMV leader of pMOGEN18 (kindly provided by Dr. B. Dekker, MOGEN International B.V., Leiden) using an *Eco*RI-*Hin*dIII adapter sequence. A full length PI2 cDNA clone, p303.51, was selected from a tuber specific *Solanum tuberosum* cv Bintje cDNA library using p303 as a probe (Jongsma *et al.*, in press; Stiekema *et al.*, 1988). PI2 cDNA clones were constructed possessing either Leu or Arg at position 5 of the mature protein. Transgenic plants described in this report have the *wt* Leu^5. Two gene fragments were generated by PCR using p303.51 as a template. The N-terminal part was amplified using 152FO: 5'-GCGGGATCCACCATGGCTGTTCA and 153BA: 5'-TGCAAGCTTTCGCATCAACAT, and the C-terminal part using 154FO: 5'-ATGCCATGGCGAAAGCTTGCACTC[G/T]AGAATGTGGTAATC and 155BA: 5'-CCGCT GCAGAGATCTTTATCACATAGCGGGGTACATATTTG). The two fragments were joined at the *Hin*dIII site, sequenced, and subcloned into the *Nco*I-*Bgl*II polylinker of pCPO33 as a transcriptional fusion with the double CaMV-35S promotor with AlMV RNA4-leader sequence and the Nos-terminator.

Binary vectors were mobilized from *Escherichia coli* strain XL1-blue (Stratagene Inc., La Jolla, USA) into the non-oncogenic *A. tumefaciens* strain GV3101 (pMP90RK) (Koncz and Schell, 1986) using a triparental mating procedure (Ditta *et al.*, 1980) utilizing the helper plasmid, pRK2013 in E. coli HB101. *Nicotiana tabacum* cv Samsun NN was transformed using a leaf disc method (Horsch *et al.*, 1985) and selected using kanamycin. After rooting, plants were transferred to the greenhouse.

Plant protein isolation and determination of PI2 concentration.

Leaves of four week old tobacco seedlings (217A, 219A, CON6) were ground to a fine powder in liquid nitrogen. The powder (16 g) was extracted with 40 ml ice-cold extraction buffer (2.5% polyvinylpolypyrrolidone; 50 mM Na_2EDTA; 0.2% phenylthiourea; 0.5% diethyldithiocarbamate; 100 mM Tris-Cl, pH 7.6) by shaking. The extraction mixture was centrifuged at 35,000xg for 10 min at 4°C. Protein of 32 ml clear supernatant was precipitated by adding solid ammonium sulphate to 80% saturation. After 1 hour on ice protein was pelleted at 15,000xg for 10 min at 4°C. Supernatant was removed twice after centrifugation. The pellet was redissolved in 10 ml distilled water with insoluble protein removed by centrifugation. Protein was stored in aliquots at -80°C.

The concentrations of trypsin and chymotrypsin solutions were determined by active site titration (Bender *et al.*, 1966; Chase *et al.*, 1967). Proteolytic enzymes (0.1 ml, 200 nM) and 0.13 ml buffer (100 mM Tris-Cl, pH 7.6) were pre-incubated for 15 min with 0-20 μl 217A (10.8 mg/ml) or 219A (8.7 mg/ml) protein extract. The volume of PI2-extract was

made up to 20 µl with CON6 (8.9 mg/ml). Reaction was started by adding 0.5 ml 10 mg/ml azocasein (Sigma) solution made up in buffer, and continued for 30 min at 37 °C. Reaction was stopped by 0.25 ml 40% TCA, and absorbance was read at 340 nm.

Insect feeding trials

Seeds of selfed transgenic tobacco genotypes were sown each week in potsoil. Twenty-four plants per genotype were transferred to 1 liter pots two weeks after sowing and grown to a 6-7 leaf stage in an additional 4-5 weeks. Plants were grown in an air-conditioned greenhouse during early spring with supplementary high pressure sodium light under a 16 h day - 8 h night photoperiodic regime. Temperatures followed the day/night rhythm with 22/18 °C and the relative humidity was kept constant at 70%.

Every day four developmentally identical plants were selected for every genotype. The top four leaves were excised at the leaf base with a razor blade and placed in an Eppendorf tube filled with 0.4% water-agar (Oxoid, purified agar). The water-agar in the tube sustained full turgor in the leaves for 24 hours. The leaves were placed in petri-dishes, which contained a piece of 2% water-agar as a humidifier and filter paper to absorb excess moisture. *Spodoptera exigua* larvae were obtained from a colony maintained on an artificial diet at IPO-DLO (Wageningen, The Netherlands). Larvae were synchronized by selecting only late first instars with head capsule slippage. Eight to nine 1st instar larvae were placed on one leaf and received fresh leaves from the same stem position of fresh, intact plants every 24 or 12 hours depending on the experiment for 6-7 days. Insects were reared in a dark cabinet at a temperature of 27°C.

Preparation of gut extracts

At day 6 and 7 larvae were weighed. Fifth instar larvae increased in weight from about 55 mg just after molting to 240 mg prior to pupation. Only larvae of more than 100 mg in weight and with full guts were selected for gut extraction. The guts of cold-anaesthetized larvae were removed, and dried on a piece of tissue paper to remove haemolymph fluid. Individual guts were weighed in tubes, frozen in liquid nitrogen and stored at -80°C.

Guts were homogenized using a plastic potter in a volume of ice-cold extraction buffer (0.9% NaCl; 5% polyvinylpolypyrrolidone; 0.5% sodium diethyldithiocarbamate) equivalent to the weight of the gut. Homogenates were clarified by centrifugation (10 min, 12.000xg, 4°C). The supernatant was transferred to a clean tube and clarified again by the same procedure. Clear green/yellow supernatants were subsequently stored at -80°C for use in activity assays.

Determination of free and inhibited proteolytic activities

Proteinase activity towards a proteinaceous substrate was measured using azocasein (Sigma, St Louis, USA) as a substrate. Gut extract (5 µl) was preincubated for 15 min at 24 °C with 0.245 ml pH10-buffer (50 mM glycine-NaOH, 5 mM $CaCl_2$; pH10). Subsequently, 0.5 ml azocasein solution (10 mg/ml in pH10-buffer) was added and incubated at the same temperature for 30 min. To stop the reaction 0.25 ml 40% TCA was added and left to stand for 15 min. Samples were centrifuged (5 min, 12,000xg) and supernatants were measured against a substrate blank in plastic cuvettes at 340 nm.

Free and inhibited trypsin-like activity was measured directly using BApNA (Benzoyl-D,L-arginine-p-nitroanilide) as a substrate applying the principle that at pH 10 proteinase and inhibitor are complexed, but that at pH 2-3 they are dissociated (Laskowski and Sealock, 1971; Green, 1953, 1983). In plastic cuvettes 5 μl gut extract was pre-incubated at 24 °C with 45 μl of pH 10-buffer or pH 2.5-buffer (50 mM glycine-HCl; 5 mM $CaCl_2$; pH 2.5). After 15 min 0.95 ml 1 mM BApNA solution (10 mM BApNA dissolved in DMSO, adjusted with 9 volumes of 10x pH 10-buffer) was added directly into the cuvette and the release of p-nitroanilide (ϵ = 8800 $cm^{-1}M^{-1}$ (Erlanger *et al.*, 1961)) was followed immediately at 405 nm (10 min, 24°C) using a thermostatted spectrophotometer (Uvikon). The rapid reassociation of inhibitors and proteinases at pH 10 induced an equivalently rapid decrease of the rates of p-nitroanilide (pNA) release. If we assume proteinases and inhibitors to be present in approximately equivalent amounts, and the complexity of the mixture of proteinases or inhibitors is not taken into account, reassociation follows second order kinetics according to the formula: $1/[P_d] = kt + 1/[P_0]$, where $[P_d]$ is the concentration of dissociated proteinase, k is the rate constant of association, t is time and, $[P_0]$ is the initial concentration of total inhibited activity (Roseveare, 1931). A graphical representation of $1/[P_d]$ vs. t should then give a straight line with slope k (Green, 1957). To measure the fraction of inhibited gut proteinase ($[P_d]$) the free proteolytic activity remaining after full complexation (obtained by a process of curve fitting) was subtracted from the observed rate of BApNA hydrolysis, which is a measure of total proteinase activity present.

In vitro inhibition of gut proteinase activity

Gut proteinase activity was inhibited *in vitro* using protein extracts from transgenic plants. Extraction of plant protein was as described above. Gut extracts of twelve guts were pooled and 5 μl of extract was added to 65 μl of pH10 buffer (100 mM glycine, 10 mM $CaCl_2$, pH10) directly in the cuvette. Protein extract from PI2-plants (0-30 μl) was added and the volume made up to 30 μl with protein extract from control plants so that any observed inhibition would be the sole effect of PI2 present in the extract. After 15 min of preincubation at 24 °C 0.9 ml 1 mM BAPNA solution (1 mM BAPNA in 1 volume DMSO, 9 volumes pH10-buffer) was added to 8 samples and measured simultaneously for 10 min at 405 nm.

Statistical analysis

All data sets were analyzed for significant differences by Student's *t*-test using the number of data mentioned in the legends of the figures.

RESULTS

Generation and characterization of transgenic tobacco plants expressing potato PI2

A cDNA clone isolated from potato encoding a member of the proteinase inhibitor II gene family was expressed in tobacco under the control of the double 35S CaMV promoter with AlMV-leader and NOS-terminator. Two initial transformants (R_0) 217A and 219A were selected for high PI2-expression using an activity assay (Jongsma *et al.*, 1993). Southern analysis and segregation ratios of kanamycin resistance revealed three (217A) or four (219A)

integrated copies of T-DNA present at several loci in the tobacco genome (not shown). Both initial transformants were selfed two (219A) or three (217A) times to obtain progeny with uniform levels of PI2-expression. R_3-progeny of 217A, and R_2-progeny of 219A and CON6, a transformed control (pCPO33 without PI2), were used for further analyses and insect feeding trials.

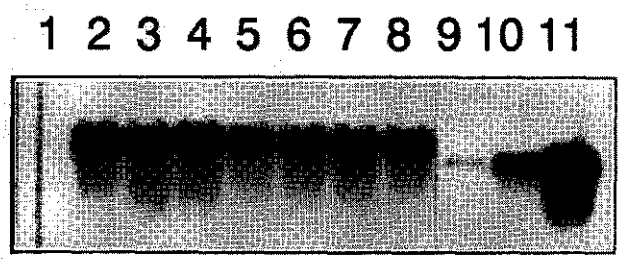

Figure 1. Northern blot of total plant RNA. Ten μg of RNA was separated using formaldehyde-agarose gels and blotted onto Genescreen Plus membranes. The blot was probed with a ^{32}P-labeled fragment of the PI2-gene. RNA was isolated (Verwoerd *et al.*, 1989) from specific leaves on a young plant numbered from top to bottom. Lane 1, leaf 3 of CON-6; lane 2-7, leaves 1-6 of 217A; lane 8, leaf 3 of 219A; lane 9-11, 5-50-500 pg *NcoI-BglII* fragment of PI2-DNA

Northern analysis detected a single mRNA species, not present in CON6, and was estimated to comprise 0.1-0.5% of total mRNA (Fig. 1). PI2-expression levels were determined by titrating the active sites of trypsin and chymotrypsin with total soluble protein. Transgenic 217A and 219A expressed 134 pmol (Fig.2) and 83 pmol/mg inhibited proteinase molecules per mg total soluble protein of additional activity relative to the controls. This amounted to approximately 0.16 % of total soluble protein in the case of 217A.

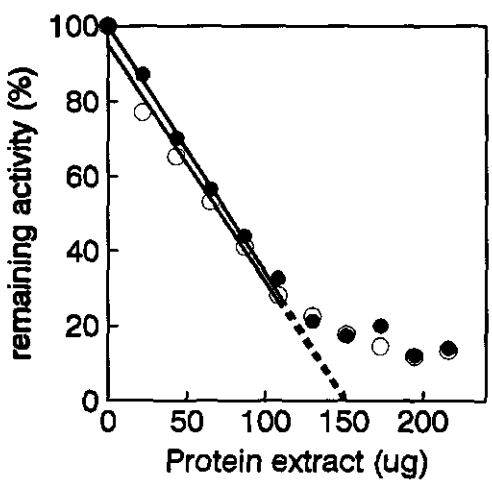

Figure 2. Titration curve of a soluble protein extract from plant 217A to determine the PI2-concentration. Trypsin (○) and chymotrypsin (●) were titrated at a concentration of 26.7 nM by increasing quantities of 217A protein extract. Linear regression curves were calculated for activities after addition of up to 100 μg protein extract. The intercept of the x-axis provided the amount of protein containing 20 pmol trypsin/chymotrypsin inhibitor domains.

Induced endogenous PI activity in tobacco leaves

The induction of endogenous trypsin inhibitor activity was assayed before and after insect feeding using the radial inhibitor diffusion assay (Jongsma *et al.*, 1993). As shown in figure 3, prior to insect feeding PI activity was only present in leaf juice of PI2-plants and not in the controls. Twenty-four hours after insect feeding commenced, PI activity was present in both control and PI2-plants. The diameter of the inhibited zones was converted on the basis of a reference line (Jongsma *et al.*, 1994) to the molar level of trypsin inhibitor activity present in the leaf (Table 1). This showed that endogenous PIs were induced by feeding larvae to similar or even higher concentrations than transgenic PI2.

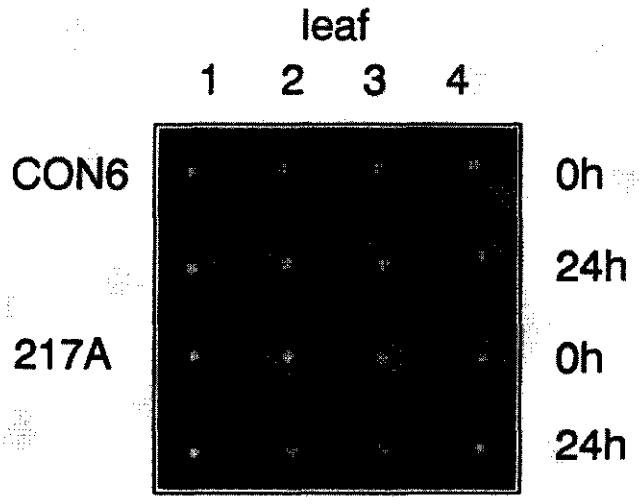

Figure 3. Trypsin inhibitor activity detected in CON-6 and 217A leaves before (0h) and after 24 h of feeding by 5th instar *S. exigua* larvae. Activity in juice pooled from four leaves was assayed by 18 h diffusion into a layer of agar containing 40 nM trypsin and was developed as described (Jongsma *et al.*, 1993). The size of the inhibited zone is proportional to the logarithm of the inhibitor concentration.

Table 1. Endogenous trypsin inhibitor (TI) concentrations induced in excised control tobacco leaves by 24 hour feeding damage

leaf # (CON6)	protein (mg/ml juice)	zone diameter (mm)	TI (pmol/mg)
1	8.2	17	170
2	6.9	18	300
3	5.9	17	240
4	4.6	16.2	240

Obviously, tobacco leaves with high induced levels of PI activity are not suitable for observing the effects of transgenic PI2 expression on larval gut physiology. PIs accumulate in tobacco leaves approximately 12 hours after wounding (Pearce *et al.*, 1993). Therefore, insects were also reared on leaves that were replaced every 12 instead of 24 hours to reduce ingestion of induced endogenous PIs, that would interfere in the study of effects caused by the transgene.

Insect feeding trials on transgenic tobacco leaves

Relative to the transformed control, CON6, no significant *S. exigua* larval weight differences were observed for either PI2-genotype, when larvae were provided with fresh leaves every 12 (not shown) or 24 hours (Fig. 4). It was observed, instead, that leaf position strongly determined larval weight (Fig. 4). The youngest leaves (leaves 1 and 2), which were not yet fully expanded, supported an average weight gain of ca. 60 mg after 6 days, but larval weights on fully expanded leaves were significantly lower (Fig. 4). On leaf three larvae attained an average weight of 40 mg ($p<0.01$, comparing leaf 3 to 2), and on leaf four 25 mg ($p<0.02$, comparing leaf 4 to 3). Growth was further inhibited on leaves 5-7 (not shown) of both control and transgenic plants. Only by comparing larval growth on leaves of identical physiological age were we able to obtain reproducible results in feeding trials.

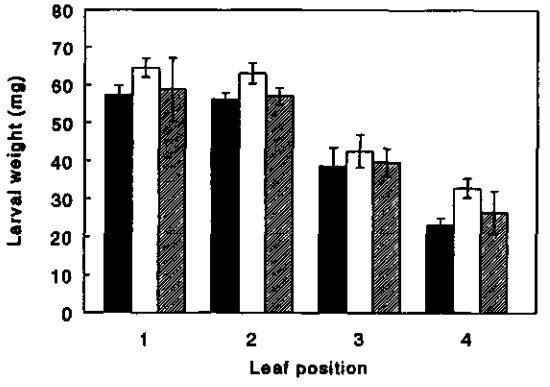

Figure 4. Average weight of larval *S. exigua* reared on excised leaves of three tobacco genotypes. First instar larvae received fresh leaves of specific stem position from fresh plants every 24 hours for 6 days. Leaves of CON6 (■), 219A (□), and 217A (▨) were numbered from top to bottom. Each data point is the mean of four excised leaf assays determining the average larval weights (8-9 larvae). Standard error bars are based on the means of the independent assays (n=4).

Proteinase activity in guts of S. exigua larvae

To study the effects of PIs on gut proteinase activity, guts of larvae chronically exposed to PI2 from first to fifth instar were dissected out. Proteinase activity of gut extracts was analyzed using a proteinaceous substrate (azocasein), and a synthetic trypsin substrate (BApNA), which is hydrolyzed by the major *S. exigua* gut proteinases (Jongsma *et al.*, in preparation). The expression of transgenic PI2 caused a reduction of azocasein digestion (Fig. 5a) and BApNA hydrolysis (Fig. 5b) of 22% ($p<0.06$) and 29% ($p<0.02$) respectively, when used leaves were replaced with fresh leaves every 12 hours. This reduction of proteolytic activity was apparently not sufficient to cause any growth retardation.

The lack of growth inhibition with *S. exigua* larvae in the feeding trials on transgenic plants expressing PI2, contrasted with the 20% reduction in growth observed using PI2 in artificial diets (Broadway and Duffey, 1986). Biochemical analyses were carried out on the guts of insects reared on the different types of leaves to investigate the physiological background of this insensitivity to PI2.

When insects were allowed to feed for 24 hours, endogenous PI activity was induced to high levels in both transgenic and control leaves. It was found, that as a consequence gut activities of insects reared on both control and transgenic leaves were lowered to the same

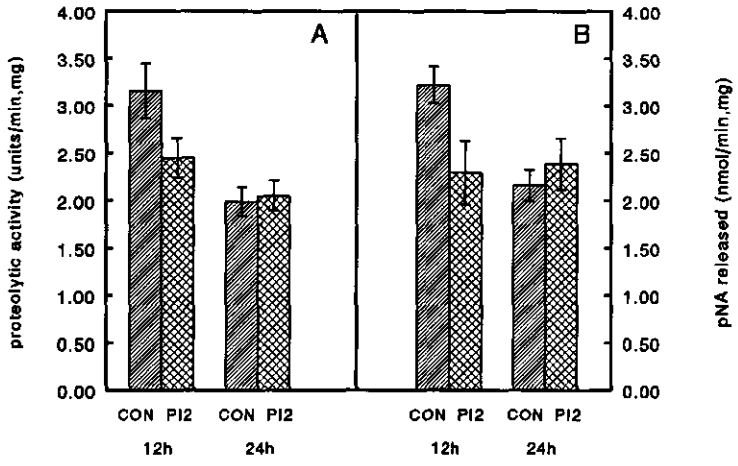

Figure 5. The rates of hydrolysis of azocasein and BApNA by gut extracts of *S. exigua* larvae. First instar larvae were fed fresh tobacco leaves from control (▨) or PI2-plants (■) every 12 or 24 hours for 6 days. **A**: Rate of proteolysis of azocasein expressed in units (1000 x OD_{340}) of TCA-soluble peptides released per min per mg gut (n=12) at pH 10. **B**: Rate of hydrolysis of BApNA per mg gut at pH 10 (number of guts: 12h: n=12; 24h: n=21,20). Variation is given as standard error bars.

extent. Azocasein digestion was reduced by 36% ($p < 0.01$) (Fig. 5a) and BApNA hydrolysis by 30% ($p < 0.03$) (Fig. 5b) relative to the control gut extract with low endogenous PI levels. The observation that the induced endogenous PIs caused the disappearance of an observable effect of transgenic PI2 on gut proteinase activities implies that the induced endogenous PIs might disguise a putative resistant phenotype of transgenic plants expressing PI2.

Composition of larval gut proteinase activity

Having established that ingested PI2 and endogenous PIs lowered insect BApNA hydrolysis and azocasein digestion by 22-36% *in vivo*, we wanted to investigate whether higher PI2-expression levels could further reduce these activities. Therefore, the composition of gut proteinase activity was determined by titrating the activities towards BApNA with crude PI2-protein extracts obtained from the leaves of transgenic plants. PI2 was found to inhibit as much as 76% of BApNA hydrolysis catalyzed by gut extracts of insects fed on control leaves with low endogenous PI levels (Fig. 6, curve A), confirming the potential of PI2 to strongly reduce proteinase activity in *Spodoptera* guts that had not been previously exposed to PIs (Broadway and Duffey, 1986). In contrast, gut proteolytic activity of insects that had been fed PI2-leaves, contained only 18% activity that could be inhibited by PI2 (Fig. 6, curve B), establishing that dietary PI2 had effectively reduced the level of free PI2-sensitive activity in the gut. However, guts of control insects contained 0.77 nmol pNA/min·mg gut of PI2-insensitive activity, while guts of insects that had been exposed to PI2 in the leaf diet contained activity equivalent to 1.88 nmol/min·mg (Fig. 8), demonstrating that the *in vivo* response of the larvae to PI2 was the induction of PI2-insensitive activity.

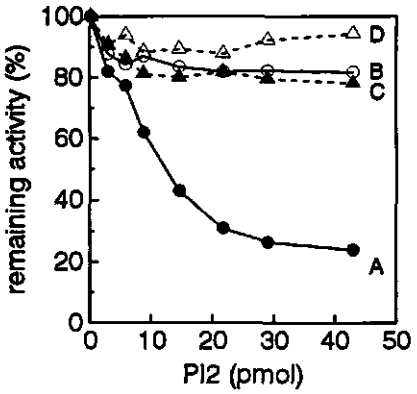

Figure 6. *In vitro* inhibition by PI2 of gut bapnase activity. Increasing amounts of a total protein extract from PI2 tobacco plants was used to inhibit the equivalent of 2.5 mg pooled (n=12) larval guts. Curves represent: Control guts of larvae reared on leaves with a low (A) or a high (C) level of endogenous PIs. PI2 gut extracts of insects reared on leaves with a low (B) or a high (D) level of endogenous PIs.

Similar to what we observed after chronic exposure to PI2, only 22% of proteolytic activity could be inhibited by PI2 in insects chronically exposed to endogenous PIs (Fig. 6, curve C). Also in this case, dietary endogenous PIs resulted in a significant increase of proteolytic activity which could not be inhibited by PI2 (0.77 nmol/min·mg to 1.68 nmol/min·mg, Fig. 8). When insects were chronically exposed to both endogenous PIs and transgenic PI2 together, only 6% of gut activity was still sensitive to PI2 (Fig. 6, curve D). In this case the induction of PI2-insensitive activity was strongest as it increased from 0.77 nmol/min·mg to 2.24 nmol/min·mg (Fig. 8). These data suggest that tobacco proteinase inhibitors and potato PI2 are active towards the same types of proteinases and that they both induce new, PI2-insensitive proteolytic activity in the gut.

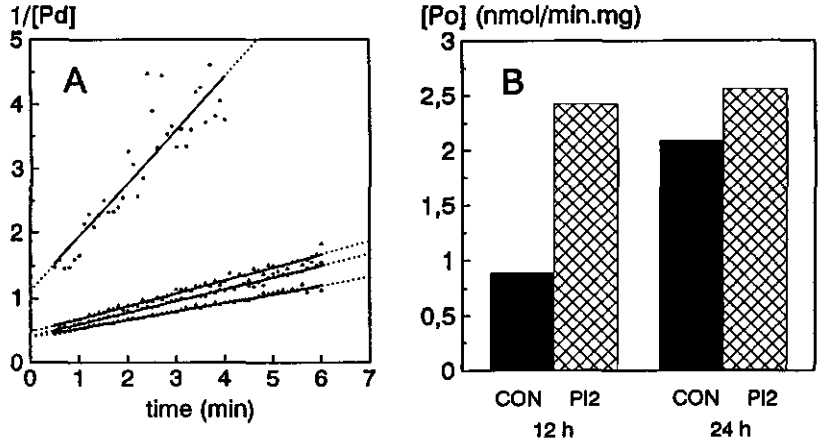

Figure 7. Determination of the concentration of inhibited gut proteinase activity. **A:** Reaction between acid-dissociated gut proteinases and proteinase inhibitors plotted according to second-order kinetics. Reaction was followed by taking the decrease in the rate of BApNA hydrolysis in time to represent the decrease in the concentration of dissociated proteinase molecules, [P_d]. Rates in the range of 30-70% complexation (Green, 1957) were used to draw the curves for the gut activities of insects reared on non-induced (○) and induced (▲) control leaves, and non-induced (●) and induced (▲) PI2-leaves. **B:** Inhibited fraction of gut bapnase activity ([P_o]) determined from the y-axis intercepts of Fig.7A (number of guts analyzed for 12h: n=10 (CON), 11 (PI2); 24h:n n=16 (CON), 17 (PI2)).

Gut proteinase activity inhibited by plant PIs

Proteinaceous serine proteinase inhibitors and serine proteinases are complexed reversibly and can be dissociated at pH < 3 (Laskowski and Sealock, 1971; Green, 1983; Green, 1953). An assay was developed to allow the calculation of the total amount of gut proteinase activity present in the gut as an inactive complex with endogenous tobacco PIs and transgenic PI2. The inhibitor-proteinase complexes were dissociated at pH 2.5, and the process of reassociation (10-20 min) was measured at the optimal pH 10. A graphical representation of the reassociation reaction according to the formula given in Materials and Methods is shown in figure 7a.

The straight curves that were obtained for the different reassociation reactions of inhibited gut activities demonstrated second order kinetics. The intercept of the y-axis ($1/[P_0]$) provided the level of proteinase activity that had been released from the inhibitor before reassociation. The y-intercept of the non-induced control was above that of the other lines, indicating that only 0.9 nmol/min·mg of the activity was already inhibited (Fig. 7b). In contrast, larvae that were fed leaves containing high levels of endogenous or transgenic PIs had levels of 2.1-2.6 nmol/min·mg of inhibited proteinase activity. Comparison to figure 5b shows that in the presence of PIs, inhibited and free activities were present in approximately equivalent amounts in the gut. Total gut proteinase activities (free plus inhibited) of larvae fed PI2-leaves were slightly higher than the controls (Fig. 8), demonstrating that *S. exigua* larvae did not respond to PIs by dramatically increasing the overall synthesis of proteinases, but appeared to have compensated for loss of activity by producing higher concentrations PI2-insensitive proteinases.

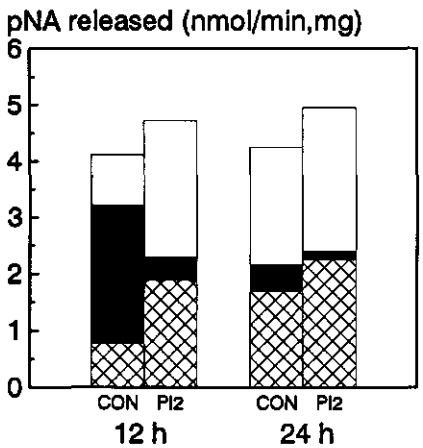

Figure 8. Composition of gut bapnase activity of *S. exigua* larvae in relation to dietary inhibitors. Guts of 5th instar larvae were analyzed as described in Fig 5B,6, and 7B after 6 days of feeding on control (CON) or PI2-leaves that were replaced every 12 or 24 hours. PI/PI2-sensitive inhibited activity (□); PI2-sensitive free activity (■); and PI2-insensitive free activity (▩).

DISCUSSION

PI2 and endogenous PIs from tobacco induce an increase in gut proteinase activity insensitive to inhibition.

In this study we demonstrate that *S. exigua* larvae adapt to chronic exposure to proteinase inhibitors by induction of new proteolytic activity which is insensitive to PI2. We found that the fraction of gut tryptic activity which is sensitive to PI2 had decreased from 76% in guts of insects unexposed to PIs to 6-22% in guts of insects that had been chronically exposed to PI2 and/or endogenous PIs. Interestingly, a different type of proteinase activity, which was insensitive to PI2 increased 2.5 to 3-fold, compensating largely, but not completely, for the lost activity. Acid-induced dissociation of inhibitor/proteinase complexes in gut extracts of larvae exposed to PIs revealed that PI-sensitive activity had not increased, but had remained constant. These results point to a mechanism of response to dietary proteinase inhibitors which depends on a repertoire of different proteinases, and is able to maintain a sufficient level of activity to allow normal growth and development. This control mechanism presumably directs the induction of gut proteinases which are insensitive to plant PIs. We have observed that also the coleopteran Colorado potato beetle induces novel PI-insensitive activity when reared on methyl jasmonate-induced potato plants with high endogenous PI levels, suggesting that this form of adaptation to plant proteinase inhibitor defense is general among insects (Bolter and Jongsma, *in press*). Also in rats and humans the induction of PI-resistant proteinases in response to inhibition by plant proteinase inhibitors has been described (Holm *et al.*, 1988, 1991). The control mechanism in the guts of rats that signals the pancreatic secretion of proteinases in response to inhibition by PIs has been shown to depend on a monitor peptide that is negatively regulated by active proteases (Iwai *et al.*, 1988).

Effects of potato PI2 expressed in transgenic tobacco on growth of S. exigua larvae

During our experiments it was found that growth of several species of lepidopteran larvae (*Manduca sexta*, *S. exigua*) were extremely sensitive to factors like leaf (Fig. 4) and plant age, plant history, and conditions of bioassay. Only when we used controlled conditions: seedlings instead of cuttings, excised leaves instead of whole plants, leaves of identical age instead of mixed ages, and when we ran several independent parallel experiments, did we obtain reliable results. Particularly relevant was our observation that endogenous PIs had been induced to levels equivalent to transgenic PI2 in (excised) leaves that had served as a food substrate for 24 hours (Jongsma *et al.*, 1994). Moreover, and in agreement with the recent characterization of these PIs as members of the PI2-family (Atkinson *et al.*, 1993; Pearce *et al.*, 1993), we demonstrated that the activity spectrum of these endogenous PIs overlapped with the activity spectrum of transgenic PI2. It was, therefore, essential to refresh leaves within 12 hours if effects of transgenic PI2 were to be investigated. However, despite considerable levels of transgenic PI2, we found that transgenic leaves fed to *S. exigua* were not effective in reducing larval growth as compared to control leaves. We think that at least two factors might account for the lack of a resistant phenotype. Firstly, as argued above, the insects are largely able to compensate for the loss of proteolytic activity by the induction of new, PI2-insensitive proteolytic activity. Secondly, although considerable levels of transgenic PI2 were obtained leading to a significant reduction of gut activity, inhibition of PI2-sensitive

proteinases was not complete (ca. 80%). It is possible that some growth retardation would be observed, if complete inhibition of this set of proteinases could be attained by higher PI2 expression levels. This suggestion is supported by the fact that high PI2-levels in artificial diets caused a growth reduction of 20% in *S. exigua* larvae (Broadway and Duffey, 1986).

We observed a reduced level of gut proteinase activity in response to the presence of proteinase inhibitors in the diet (Fig. 5). Using specific synthetic substrates others also observed that insects fed PIs mixed in artificial diets had lower levels of gut proteinase activity (Burgess *et al.*, 1994; Johnston *et al.*, 1993; Orr *et al.*, 1994). In contrast to these observations, Broadway and Duffey (1986) and Burgess *et al.* (1991) did not observe a reduced level of gut proteinase activity, but instead found that gut activities were even slightly increased. From this it was concluded that the growth inhibition could have resulted from a pernicious hyperproduction of proteinases which depleted the amino acid pool, and not from an actual reduction of protein digestion as suggested by Reese (1983). However, caution should be exercized with the interpretation of activities towards specific synthetic substrates. We described that after chronic exposure to PIs guts had completely altered proteinase compositions. These different proteinases can have widely differing rates of hydrolysis for different *synthetic* substrates (Holm *et al.*, 1991), that do not reflect physiologically relevant increases or decreases in digestion of protein. The hydrolysis of azocasein, a general proteinaceous substrate, may more accurately describe the effects of PIs on gut protein digestion. In this report, the observed reduction *in vivo* of the hydrolysis of azocasein indicates that PIs can reduce the digestion of food proteins. Even though, in our case it was apparently insufficient to affect larval growth, it may indicate that PIs primarily affect growth by reducing protein digestion and the concomitant reduced availablity of free amino acids.

Consequences for the application of PIs in transgenic plants to obtain insect resistance

Transgenic tomato plants with the systemin gene transcribed in the antisense orientation have been reported to have a repressed endogenous PI induction in response to wounding (McGurl *et al.*, 1992). In insect feeding trials these plants have been demonstrated to sustain higher growth rates of *Manduca sexta* larvae than wild-type plants (Ryan *et al.*, 1993). This provided evidence that plants are already partially protected by the naturally induced PI defense. Constitutive expression of high levels of transgenic PI genes could enhance this natural protection an additional 50% in terms of larval weights (Hilder *et al.*, 1987; Johnson *et al.*, 1989; McManus *et al.*, 1994). In combination, transgenic and endogenous PIs probably delay insect development by several days, but rarely is their survival affected. In this study, we demonstrated that a possible reason for the relative resistance of insects to PIs is the induction of PI-insensitive proteinase activity upon exposure to PIs. This activity allows them to digest protein independent of the level of (ineffective) proteinase inhibitors. This finding implies, that strategies aiming to find or design inhibitors specifically directed against PI-insensitive proteinases complementary to the endogenous PIs in a particular plant background, may lead to a more successful application of PIs in transgenic plants as an approach to insect resistance (Jongsma *et al.*, 1995).

CHAPTER 5

CHARACTERIZATION AND PARTIAL PURIFICATION OF GUT CYSTEINE AND SERINE PROTEINASES OF *SPODOPTERA EXIGUA* (HÜBNER) (LEPIDOPTERA: NOCTUIDAE)

Proteolytic activity present in the luminal contents of larval guts of the beet army worm, *Spodoptera exigua* (Hübner), was characterized. Separation of gut proteins by SDS-PAGE and subsequent incubation with proteinase substrates visualized six separate activities, with apparent molecular masses that ranged from 29 to 110 kDa. Pre-incubation of the gel with specific low molecular weight inhibitors revealed the specificities of the separated proteinase activities. Five activity bands behaved as serine proteinases, but a sixth band, P31, responsible for 21% of total proteinase activity appeared to have cysteine proteinase activity. P31, P35 and P45 were subsequently partly purified by anion exchange chromatography and further characterized with specific inhibitors and substrates.

INTRODUCTION

The beet army worm, *Spodoptera exigua* (Hübner), is a widespread and very polyphagous lepidopteran pest causing severe economic damage in both dicotyledon (sugar beet, alfalfa, cotton, chrysanthemum) and monocotyledon crops (rice)(Hill, 1987). A possible approach to control this insect is with the use of plant genes encoding proteinase inhibitors (PIs) in transgenic plants. Plant PIs have been shown to inhibit gut proteinases of insects and stunt the growth of larvae, when mixed in artificial diets (Broadway and Duffey, 1986; Johnston *et al.*, 1993; Burgess *et al.*, 1994; Orr *et al.*, 1994; Oppert *et al.*, 1993), or when expressed in transgenic plants (Hilder *et al.*, 1987; Johnson *et al.*, 1989). The delay of larval development achieved by individual PIs is mostly not very large. It has been suggested that it will require multiple PIs that are effective against all major activities in the gut to provide effective protection (Gatehouse *et al.*, 1993; Jongsma *et al.*, 1995b). As a first step to achieving this it is necessary to characterize gut proteolytic enzymes.

Proteolytic enzymes can be classified into four different classes based on the amino acid or metal ion involved in the catalytic hydrolysis of the peptide bond: (i) serine proteinases, (ii) cysteine proteinases, (iii) aspartic proteinases, (iv) metallo-proteinases. Proteinase activities in crude extracts of guts of different species of lepidopteran larvae have been characterized using class-specific proteinase inhibitors (Broadway, 1989; Purcell *et al.*, 1992; Wolfson and Murdock, 1990; Ahmad *et al.*, 1976; Christeller *et al.*, 1992; Houseman *et al.*, 1989; Ishaaya *et al.*, 1971; Pritchett *et al.*, 1981). In some cases proteinases have been purified (Miller *et al.*, 1974; Johnston *et al.*, 1991; Milne *et al.*, 1993; Xu and Qin, 1994; Ahmad *et al.*, 1980; Eguchi and Kuriyama, 1985), and sequenced at the amino acid (Sasaki *et al.*, 1993) or DNA level (Peterson *et al.*, 1994, 1995). Gut proteinase activities

of different *Spodoptera* species have been shown to have pH optima of 10-11, molecular weights between 17-53 kDa, and to be inhibited by serine proteinase inhibitors (Ishaaya *et al.*, 1971; Ahmad *et al.*, 1976, 1980; Lee and Anstee, 1995).

In this report the characterization and partial purification of gut proteinase activity of 5th instar *Spodoptera exigua* larvae is described. Evidence for both serine and cysteine proteinases is presented.

MATERIALS AND METHODS

Abbreviations

APNE, N-acetyl-DL-phenylalanine-β-naphtyl ester; BANA, Nα-Benzoyl-DL-arginine-β-naphthylamide; BA*p*NA, Nα-Benzoyl-L-Arg-*p*-nitroanilide; BTEE, N-Benzoyl-L-tyrosine-ethyl-ester; cystatin, cysteine proteinase inhibitor from egg white; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); E-64, trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane; IAA, iodoacetic acid; NEM, N-ethyl-maleimide; pHMB, *p*-hydroxy-mercuribenzoic acid; PI1 (Calbiochem), potato proteinase inhibitor I; PI2 (Calbiochem), potato proteinase inhibitor II; PMSF, phenylmethylsulfonyl fluoride; SAAPL*p*NA, N-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide; SBBI, soybean bowman birk inhibitor; STI, soybean trypsin inhibitor.

Materials

Unless otherwise noted all chemicals were purchased from Sigma Chemical Co. (St Louis, Mo).

Rearing of larvae

Spodoptera exigua (Hübner) larvae were obtained from a colony maintained on artificial diet at IPO-DLO (Wageningen, The Netherlands). Field collected individuals were introduced into the colony each year to maintain genetic diversity. After hatching, all larvae used in the experiments were reared on artificial diet in plastic cups at 27°C in a dark cabinet. The artificial diet was prepared as follows: A 3% agar solution was prepared in 1 L water by heating. After cooling to 60 °C the solution was mixed with 160 g cornmeal, 80 g wheat germ, 80 g yeast flakes, 8 g ascorbic acid, 2 g sorbic acid, 1 g p-hydroxy benzoic acid, 0.1 g streptomycin and left to solidify.

Extraction of guts

Whole insects were weighed and guts were removed from 5th instar larvae as described (Broadway *et al.*, 1986). Guts were extracted by stirring for 2 h in 5 ml of distilled water. Empty guts were removed by centrifugation (12,000xg, 15 min) and the supernatant containing gut luminal contents was incubated overnight at room temperature to promote degradation of dietary protein without effects on total proteinase activity (Ahmad *et al.*, 1976). Extracts were recentrifuged (12,000 g, 10 min), sterilized using a 0.22 μm filter and stored at -20 °C until further use.

Proteinase assays

Proteinase activity was determined using [^{14}C]methemoglobin. [^{14}C]Methemoglobin was purchased in labeled form (NEN, Du Pont de Nemours), or labeled using [^{14}C]formaldehyde (NEN, Du Pont de Nemours) and sodium cyanoborohydride according to the method of Dottavio-Martin and Ravel (1978). The assay was based on Wolfson and Murdock (1990). Samples of gut extract (10 μl) were pre-incubated with inhibitor (1 μl) in buffer (40 μl, see below) for 30 min at 37^{0}C, and then 50 μl substrate solution (2 mg/ml [^{14}C]methemoglobin of specific activity 2400 Bq/mg) was added. Samples were incubated at 37^{0}C for 10-180 min depending on the time needed to obtain half-maximal degradation, at optimal pH, without inhibitors. The reaction was stopped by the addition of 0.1 ml 10% TCA on ice. After 20 min, the precipitates were sedimented by centrifugation (12,000xg, 5 min) and 0.15 ml supernatant was counted in scintillation counting vials containing 1 ml distilled water and 4 ml Ultima Gold XR scintillation fluid (Packard Instruments Co. Inc., USA). The fraction of [^{14}C]methemoglobin degraded was calculated by dividing observed counts by total counts (no addition of TCA), after subtraction of background counts (addition of TCA prior to the addition of substrate). The molar rate of degradation was calculated assuming a molecular mass of 64.5 kDa.

The following buffers were used for the determination of the activity at different pH values: pH 2.0-3.0, 200 mM glycine-HCl; pH 3.5-4.5, 200 mM β-alanine-HCl; pH 5.0-5.5, 200 mM sodium acetate; pH 6.0-7.5, 100 mM sodium phosphate buffer; pH 8.0-8.5, 100 mM Tris-HCl; pH 9.0-10.5, 200 mM glycine-NaOH; pH 11.0-12.0, 100 mM trisodium phosphate-NaOH. All buffers were adjusted to the conductivity of 150 mM NaCl. A reducing agent, 5 mM L-cysteine, was added to enhance the activity of cysteine proteinases, except in the presence of inhibitors.

For the determination of the specificity of purified proteinases, synthetic substrates were used. Purified enzyme (0.01 ml) was pre-incubated for 10 min with 0.09 ml buffer (100 mM glycine-NaOH, 10 mM $CaCl_2$, pH 10), and then 0.9 ml substrate solution was added. Substrate solutions were: (i) 1 mM BTEE (N-Benzoyl-L-tyrosine ethyl ester), 75 mM glycine-NaOH, 7.5 mM $CaCl_2$, pH 10, 25% methanol; (ii) 1 mM BApNA (Nα-Benzoyl-L-Arg-*p*-nitroanilide), 10% DMSO, 90 mM glycine-NaOH, pH 10; (iii) 1 mM SAAPLpNA (N-Succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide), 10% DMSO, 90 mM glycine-NaOH, 9 mM $CaCl_2$, pH 10. The increase in extinction was monitored at 256 nm for BTEE and 410 nm for BA*p*NA and SAAPL*p*NA. The activity of the purified proteinases towards the general proteinaceous substrate, azocasein, was also measured. Purified enzyme (0.01 ml) was pre-incubated for 10 min with 0.24 ml 100 mM glycine-NaOH, 10 mM $CaCl_2$, pH 10 and then 0.5 ml azocasein solution (10 mg/ml azocasein in buffer) was added and incubated for 3 h at 37^{0}C. Reaction was stopped by the addition of 0.25 ml 40% TCA. After centrifugation TCA-soluble peptides were measured at 340 nm.

Characterization after gel electrophoresis

Protein extracts were dissolved in non-reducing sample buffer (2% SDS, 20% glycerol, 200 mM Tris-Cl, pH 6.8, 0.1% bromephenolblue). To prevent irreversible inactivation of the proteinases, samples were not boiled, and were separated by electrophoresis on 10% SDS-polyacrylamide gels at 4^{0}C, 200 V for 1 h (Laemmli, 1970). The gel was washed twice with water and strips were incubated for 30 min in a horizontally rolling, 10 ml reaction tube

containing 1 ml of a solution of 100 mM glycine-NaOH, pH10 plus or minus inhibitors at the concentrations listed in table 1. Subsequently, a 2% casein solution prepared in the same buffer was added and incubated for 60-90 min (Garcia-Carreño *et al.*, 1993). Gels incubated in casein were subsequently coloured in Coomassie Brilliant Blue. After decoloration white bands in an otherwise blue background indicated active proteinases. Alternatively, gels were immersed in either a solution of 24 mg N-Acetyl-DL-phenylalanine-β-naphthyl ester (APNE) and 12 mg Fast Blue B (Fluka, Switzerland) or 30 mg BANA (Benzoyl-DL-arginine-β-naphthylamide) and 20 mg Fast Blue B per 50 ml solution of pH 7.6 prepared as described by Jongsma *et al.* (1993), to visualize the bands active after incubation with inhibitors.

Column chromatography

Typically 0.5-1 mg of gut extract diluted in an equal volume of 40 mM Tris-Cl, pH 7.5 buffer was applied to a Mono Q HR 5/5 (Pharmacia LKB Biotechnology) column. The column, held at room temperature, was washed with 20 mM Tris-Cl, pH 7.5 and eluted with an NaCl gradient (Fig. 4). Fractions (2.5 ml) were collected and placed on ice. Protein concentration was measured at 280 nm and the enzyme activity of each fraction was determined using azocasein as described above. The fractions of each activity peak were checked for purity on an activity gel. Pure fractions were pooled, concentrated using a 5 kD filter unit (Centrisart I from Sartorius AG, Göttingen, Germany) and stored at -20 °C.

RESULTS

pH dependence of gut proteinase activity

With [^{14}C]methemoglobin as a substrate, the pH optimum of crude *S. exigua* gut proteinase activity was found to be maximal at the highest pH range (11.5-12) investigated (Fig. 1). However, subsequent experiments with inhibitors which required a 30 min incubation time without substrate (not shown) demonstrated that at pH 12 the proteinases are not stable due to (auto)proteolysis or chemical inactivation (Ahmad *et al.*, 1980). Indeed, after partial purification, pH optima of individual enzymes were found to be in the range of

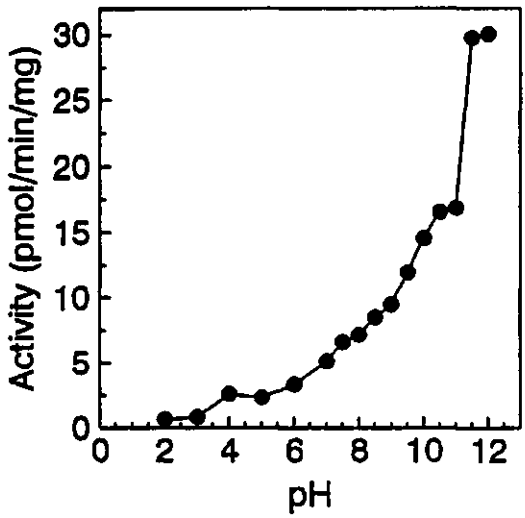

Figure 1. The effect of pH on proteolysis of [^{14}C]methemoglobin by larval *S. exigua* gut contents. Extracts were incubated with substrate solution for 7.5 min at 37°C. Buffers used are listed in Materials and Methods.

pH 10-11 (Fig. 6), corresponding to values found by others for *Spodoptera* species (Ishaaya et al., 1970; Ahmad et al., 1980; Lee and Anstee, 1995). In crude measurements the average pH in guts of *Spodoptera* species was found to vary from pH 8.2-8.5 (Berenbaum, 1980), and it seems biologically more relevant to investigate crude proteinase activity at the physiological pH of the gut. However, it has been demonstrated that the local pH along the midgut of lepidopteran insects is not constant, but differs from the average value of the whole gut by more than 2 pH units (Dow, 1992). Therefore, subsequent experiments were carried out at pH 10.

Inhibition of protease activity by specific inhibitors

Crude gut extracts were incubated with high concentrations of proteinase inhibitors specific for different classes or subclasses of proteinases. The percentage of inhibition of methemoglobin digestion provided an indication of the relative contribution of the inhibited (sub)class of proteinase to total gut proteinase activity (Table 1).

Table 1. *Effect of inhibitors on [^{14}C]methemoglobin hydrolysis*

Inhibited class	Inhibitor[1]	Concentration (μM)	crude extract[3] (% control)	purified P31[3] (% control)
serine proteinases	SBBI	12	28	19
	Aprotinin	5	34	
	STI	2.5	39	43
	PI2	4	49	15
	PI1	6	55	
	PMSF/L-Cysteine	1000/5000	83	72
	Benzamidine	1	86	
cysteine proteinases	DTNB	5000	76	35
	E-64	10	79	38
	pHMB	100	80	80
	NEM	5000	91	
	IAA	5000	96	
	Cystatin	10	97	89
	L-cysteine[2]	5000		81
aspartic proteinases	Pepstatin	10	108	
metallo proteinases	1,10-Phenanthroline	10,000	89	
serine/cysteine proteinases	chymostatin	100	12	10
	antipain	100	34	
	leupeptin	100	42	
	PMSF	1000	60	50

[1] see M&M for abbreviations
[2] stimulates cysteine proteinase activity
[3] duplicates varied by less than 5% in all cases

The proteinaceous serine proteinase inhibitors inhibited gut activity by 45-72%, demonstrating that *S. exigua*, like all other lepidopteran larvae tested so far, mainly employs serine proteinases for proteolysis. The differences in effectiveness between the inhibitors is probably due to the different specificities of these inhibitors for subclasses of serine proteinases. Synthetic serine proteinase inhibitors, like PMSF in combination with L-cysteine (PMSF without reductants will also inhibit cysteine proteinases (Dunn, 1989)) and benzamidine, were less active than the proteinaceous inhibitors.

The cysteine proteinase inhibitors cystatin and IAA were not very potent inhibitors of *S. exigua* gut proteolytic activity, but DTNB and the highly specific cysteine proteinase inhibitor, E-64, isolated from *Aspergillus japonicus* (Hanada *et al.*, 1978) decreased the activity with 24% and 21%, respectively, suggesting that cysteine proteinase-like activity contributed significantly to total activity (Table 1). Only a small fraction of the proteinase activity (11%) was inhibited by 1,10-phenanthroline and may possibly be attributed to metallo-proteinase-like activity, but 1,10-phenanthroline, which chelates divalent metal ions, may also reduce the activity of other classes of proteinases. Pepstatin did not reduce proteolytic activity, indicating that aspartic proteinases are not active in *S. exigua* guts. Chymostatin, which inhibits both serine and cysteine proteinases, was the most effective single inhibitor of gut proteolytic activity (88%).

Characterization of proteinase activities after gel electrophoresis

Individual proteinase activities separated on SDS-polyacrylamide gels were visualized by incubating gels in a protein substrate solution or in the synthetic substrates APNE or BANA (Fig. 2). APNE and BANA are substrates for both serine and cysteine proteinases

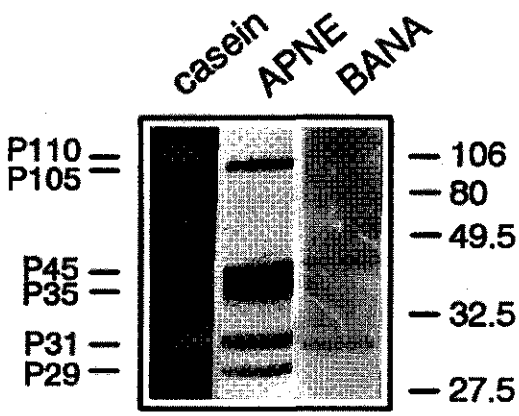

Figure 2. Detection of separated S. exigua proteinase activities after SDS-PAGE by diffusion of casein, APNE, or BANA into the gel. On the left the main activities are indicated, and on the right the molecular weight markers (kDa).

Jongsma *et al.*, 1993), but hydrolysis of APNE is optimal with proteinases that are specific for aromatic residues, such as chymotrypsin, whereas BANA is hydrolyzed by proteinases specific for basic residues, such as trypsin. *S. exigua* gut proteinases were active after SDS-PAGE electrophoresis without the recommended Triton-X resolubilization step (Garcia-

Carreño *et al.*, 1993), provided that the samples were not inactivated by heating. After incubation with casein, four major bands of proteinase activity were visible. These enzymes had apparent molecular masses of 31 kDa (P31), 35 kDa (P35), 45 kDa (P45), and 110 kDa (P110), respectively (Fig. 2, first lane), but, as the proteinases remained active even during the running of the gel, as evidenced by smeared protein degradation in substrate gels (data not shown), these values most likely do not accurately represent the molecular weights. All activities, except for activity P110, were reproducibly found with different gut extracts. The substrate APNE was rapidly hydrolyzed by five separate activities in the gel (Fig. 2, second lane). Bands P31, P35 and P45, which were active towards casein, were also active towards APNE in contrast to P110. Two additional activities, of M_r 29 kDa (P29) and 105 kDa (P105) were not visible on the casein gel, but were highly active towards APNE. BANA was only hydrolyzed by activities P31 and P45 (Fig. 2, third lane), but this activity was very light and other bands may be there.

Gels were incubated with proteinase inhibitors prior to the incubation with the substrates casein and APNE to identify inhibited proteinases directly (Fig. 3). Chymostatin, which inhibited 89% of total activity with [^{14}C]methemoglobin as a substrate, inhibited all different activities observed using casein as a substrate, but not the activities of P29 and P105 observed using APNE. PMSF (40% inhibition of crude extract) inhibited activity P35, and

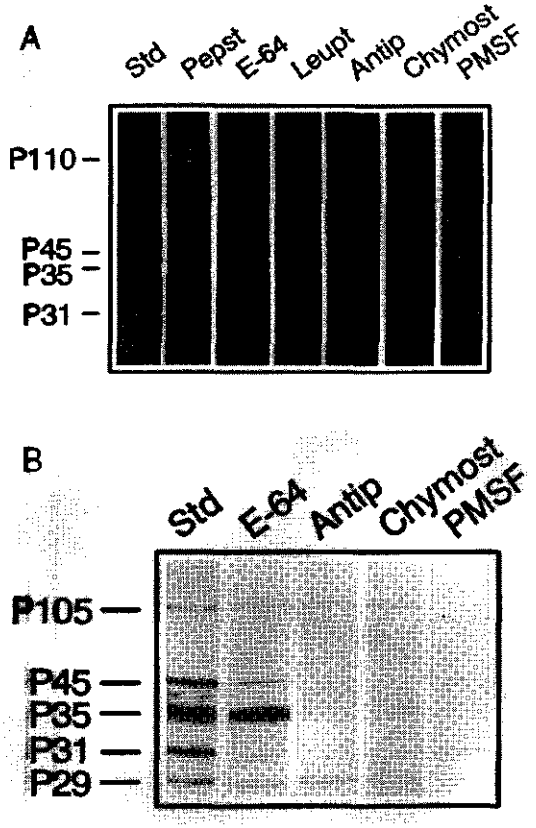

Figure 3. Inhibition of *S. exigua* gut proteinase activities by low molecular weight proteinase inhibitors. Prior to incubation in substrate solutions of casein (A) or APNE (B) gels were incubated with proteinase inhibitors at the concentrations indicated in table 1. (Abbreviations: Std, standard; Pepst, pepstatin; Leupt, leupeptin; Antip, antipain; Chymost, chymostatin)

also the P29 and P105 activities, that were only observed using APNE as a substrate (Fig. 3b). The observation that PMSF inhibits these latter two activities identifies them as serine proteinases rather than esterases that may also hydrolyze this type of substrate. Antipain and leupeptin inhibited P31, P45 and P110, which corresponded to the high level of inhibition (66% and 58%) of crude extract. E-64 inhibited only activity P31, and none of the other activities, demonstrating that the previously observed inhibition (21%) of crude proteinase activity was due to a single type of cysteine proteinase activity present in *S. exigua* guts in addition to serine proteinases. It cannot be excluded however that this P31 consists of several isozymes specific for either APNE or BANA.

In this assay proteinaceous proteinase inhibitors like SBBI and STI were not very effective in inhibiting proteinase activity, presumably, because they were used at relatively low concentrations and they will diffuse into the gel only slowly. To assess the inhibition by proteinaceous inhibitors, it was necessary to purify the different proteinase activities.

Purification and characterization of gut proteinases

Proteolytic enzymes of *S. exigua* were purified by anion exchange chromatography. The major gut proteinases were absorbed to a Mono Q HR column at pH 7.5, and separated by salt gradient elution (Fig. 4). Five major peaks of proteinase activity were detected using azocasein as a substrate (Fig 4, peaks A to E).

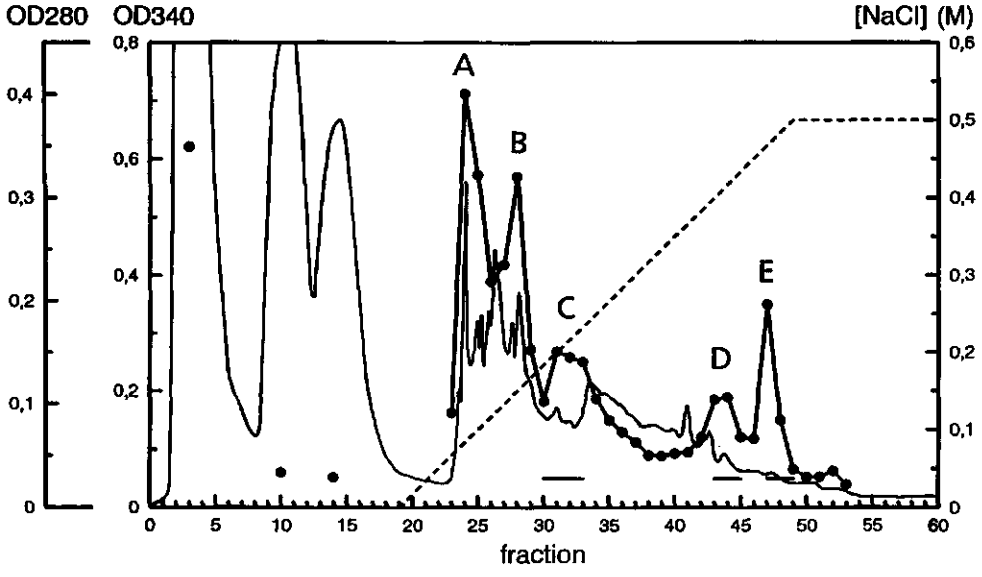

Figure 4. Mono Q HR 5/5 anion exchange chromatography of 0.5 mg crude extract of larval *S. exigua* gut contents dialyzed against 25 mM Tris-Cl, pH 7 buffer. The protein content of fractions eluted by a NaCl gradient (--) was measured by absorbance at 280 nm (—), and enzyme activity by proteolysis of azocaseine by absorbance at 340 nm (-•-).

After SDS-PAGE analysis the first two peaks (A and B) were found to represent mixtures of activities P31, P45, and P110 (not shown) and were not used for further characterization. It may indicate that several isozymes with different isoelectric points run at identical positions on substrate gels. Peaks C to E corresponded with activities P31, P35, and P45, respectively, as demonstrated by substrate-gel analysis (Fig. 5). Fractions corresponding to these peaks were pooled, concentrated, and used for further enzymatic analysis.

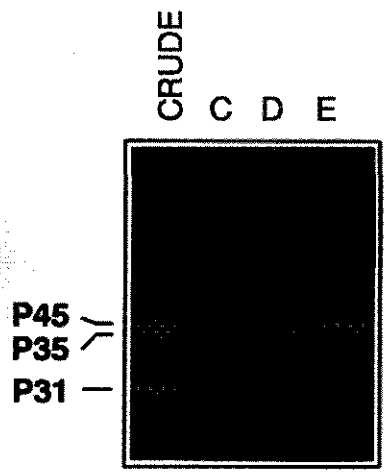

Figure 5. Detection of *S. exigua* proteinase activities partially purified by anion exchange chromatography in SDS-PAGE gels using casein as a substrate. Crude, crude gut extract; lane C, partially purified P31; lane D, partially P35; lane E, partially purified P45

After purification P31 consisted of 46% E-64 and 44% PMSF-inhibitable activity, while P35 and P45 only had 6 to 10% E-64 and 91% PMSF-inhibitable activity (Table 2). It is shown in Fig. 3, that, before purification of the mixture of proteinases, E-64 and PMSF nearly fully inhibited P31 and P35, respectively. If the degree of inhibition by E-64 and PMSF is taken as a measure of purity, then P31 was 46% pure, and P35 and P45 were 91% pure. The partial purity of P31 is not directly evident from the casein gel analysis, but this may be caused by the fact that not casein but hemoglobin was used as a substrate in the inhibitor analysis of P31. To our surprise purified P45 could be almost fully inhibited by PMSF. This suggests that crude P45 (Fig. 3) consists of several proteinases with different susceptibilities for PMSF inhibition and explains the reduced intensity of P45 in the presence of PMSF, when APNE was used as a substrate (Fig. 3b).

The pH optima of the three partially purified proteinase activities were determined for the pH range 8-12 (Fig. 6). It was found that the optima for all three purified proteinase fractions ranged between pH 10-11, corresponding with the pH optima that were reported for guts of other Spodoptera species (Ishaaya *et al.*, 1971; Ahmad *et al.*, 1976, 1980; Lee and Anstee, 1995). P31 had the sharpest optimum at pH 11 loosing a significant degree (75%) of activity at pH 12.

The purified proteinase activities were analyzed for substrate specificities using BA*p*NA, SAAPL*p*NA, and BTEE (Table 2). These substrates differ in the type of amino acid residue (arginine, leucine or tyrosine) that must be hydrolyzed and are widely used to differentiate the specificities of proteolytic enzymes. The purified proteinase activities were obtained in such low amounts, that no confident measurement of the protein concentration could be made. For that reason, activities with different synthetic substrates were expressed

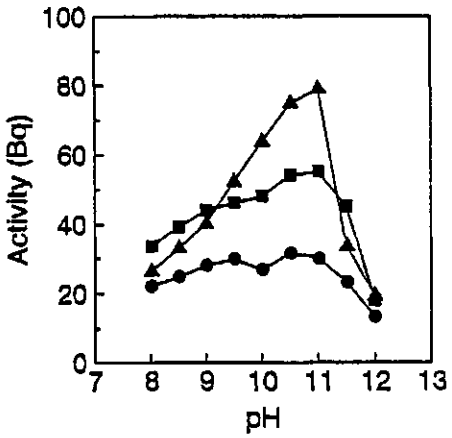

Figure 6. pH profile of the purified enzyme fractions. Proteolytic activity of 15-20 µl of concentrated enzyme fractions (0.5-1 ml) collected after anion exchange chromatography towards [^{14}C]methemoglobin was measured after an incubation of 3 h at 37°C. P31 (▲), P35 (■) and P45 (●).

relative to the activity towards the substrate azocasein, which is a general protein substrate for all three proteinases. The activities of P35 and P45 were then expressed as a percentage of P31 to facilitate a direct comparison of substrate specificities. All three purified fractions exhibited similar rates of BA*p*NA-hydrolysis. SAAPL*p*NA was hydrolyzed about 5-6 times better by P35 and P45 compared to P31, whereas P31 was around 10 times more efficient than the other two at hydrolyzing BTEE. These results demonstrate that BA*p*NA is a general substrate for the three purified *S. exigua* gut proteinase fractions, but that they differ in their specificity for aromatic (mainly hydrolyzed by P31) or neutral (mainly hydrolyzed by P35, P45) amino acid residues. This range of activities in a single proteinase is unusual and may indicate the presence of several isozymes, which could not be resolved by column chromatography or visualized by substrate gel analysis.

Table 2. Sensitivity of purified gut proteinases to inhibitors and relative enzymatic activities towards specific substrates.

	Activity		
	P31	P35	P45
Inhibitors ([^{14}C]methemoglobin)[1]			
E-64	54	94	90
PMSF	66	9	9
Substrates[2]			
Azocasein	100	100	100
BA*p*NA	100	77	200
SAAPL*p*NA	100	652	484
BTEE	100	8	12

[1] Activity is given as a percentage of the control. The pooled fractions tested here were from a different run compared to Table 1

[2] Activities are expressed as relative percentages after normalization relative to azocasein and pool I

As measured by E-64 inhibition, P31 was obtained 62% pure in a different purification (Table 1). This purified activity was characterized with a range of other inhibitors that were also used to characterize the crude extracts (Table 1). The cysteine proteinase inhibitor, DTNB, inhibited a similar amount of activity of P31 compared to E-64 (65%), while cystatin inhibited only moderately (11%). These results provided strong evidence for cysteine proteinase activity. However, typical serine proteinase inhibitors, like SBBI and PI2, also inhibited major fractions of P31 activity, and P31 was not stimulated by L-cysteine as is often observed with oxidized cysteine proteinases.

DISCUSSION

Six distinct proteinase activities from the gut of larvae of *S. exigua* were observed after SDS-gelelectrophoresis. Their apparent molecular masses ranged from 29 to 110 kDa. Proteinases were characterized in gel by pre-incubation with low molecular weight inhibitors prior to the addition of proteinaceous or synthetic substrates. The standard proteinase literature takes inhibition by E-64 as diagnostic of cysteine proteinases (Hanada *et al.*, 1978; Rich, 1986; Dunn, 1989). On this basis, it appears that P31 is a cysteine proteinase responsible for 21% of total proteinase activity. The five other activities are most likely serine proteinases. Metallo and aspartic proteinases were not significantly contributing to the protein digestion by *S. exigua* larvae.

Lee and Anstee (1995) recently reported the partial purification and characterization of a trypsin and a chymotrypsin-like activity from the guts of *Spodoptera littoralis* larvae. The trypsin-like activity could also be inhibited by E-64 and p-chloro-mercuri-phenylsulfonic acid which are diagnostic of cysteine proteinases. Those authors argued, however, that classification as a serine proteinase was more likely because, firstly, a number of insect gut proteinases of the serine type have an unusual cysteine residue in the active site (Peterson *et al.*, 1994) and are sensitive to mercurials (Sasaki *et al.*, 1993; Amarant *et al.*, 1991), while, secondly, the enzyme was relatively insensitive to E-64. We prefer to maintain a preliminary classification of P31 as a cysteine proteinase for the following reasons. Recently, Peterson *et al.* (1995) reported the characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of Manduca sexta. This serine proteinase was also sensitive to a typical sulfhydryl agent like IAA, but did not possess a cysteine residue in the active site, nor did it react with E-64. It is not the presence of a free cysteine in the active site of a serine proteinase, therefore, which can render these types of proteinases sensitive to sulfhydryl agents, as has been suggested by Lee and Anstee (1995). The fact that the sensitivity to IAA was not correlated with a sensitivity to E-64 (Peterson *et al.*, 1995), also, maintains the validity of E-64 for use as a diagnostic of cysteine proteinases. The second argument related to the relative insensitivity to inhibition by E-64 (200 μM for full inhibition). Lee and Anstee (1995) carried out their inhibitor experiments at neutral pH. We found that at neutral pH E-64 was not effective against P31 (data not shown). Only when we pre-incubated E-64 with P31 at pH 10 we could obtain full inhibition at low E-64 concentrations (< 10 μM). A similar pH dependence for inhibitors was also demonstrated by Peterson *et al.* (1995). Given the high sensitivity of P31 to inhibition by E-64, and the absence of strong evidence in the literature that other classes of proteinases can be fully inhibited by E-64, resolves suggests that P31 is more likely a cysteine proteinase. It will be of interest, however, to obtain the protein sequence of P31 to carry out a definite classification and study the unusual properties of this proteinase.

The cysteine proteinase, P31, and two serine proteinase activities (P35, P45) were purified and characterized further. Characterization with synthetic substrates demonstrated that P31 was more specific for a substrate with an aromatic amino acid (tyrosine), whereas, in contrast, P35 and P45 were more effective towards a substrate with a neutral amino acid (leucine). All purified enzymes appeared to be broadly specific for basic residues (arginine). The broad substrate specificity is rather unusual and may indicate the presence of several isozymes or copurification of proteinases that do not show up on the substrate gel. Characterization of P31 after purification demonstrated that it could be equally well inhibited by the synthetic, low molecular weight cysteine proteinase inhibitors, E64 and DTNB, but much less so by chicken egg white cystatin. Possibly, this is caused by the fact that proteinaceous inhibitors of proteinases can be highly specific for subclasses of proteinases, whereas the synthetic inhibitor, E-64, is known, to attach covalently to the thiol group of almost all known cysteine proteinases (Rich, 1986). The identity of P31 as a cysteine proteinase seems challenged by the fact, that it was not stimulated by the presence of L-cysteine, and was also inhibited by commercial proteinaceous serine proteinase inhibitor preparations like PI2 and SBBI. The lack of stimulation of the enzyme by L-cysteine may be caused by the fact that the high pH at which the assays were performed caused an effect similar to the addition of reducing agents (Creighton *et al.*, 1995) or that the enzyme was not in its oxidized state for other reasons. Inhibition of cysteine and even aspartic proteinases by commercial proteinaceous serine proteinases was also observed by Wolfson and Murdock (1990) in larval guts of *Trichoplusia ni*. They found 46% inhibition by E-64 indicating cysteine proteinase activity, but at the same time 86% (lima bean inhibitor) to 91% (soybean trypsin inhibitor) inhibition by typical commercial proteinaceous serine proteinase inhibitors. These proteinaceous inhibitor preparations were also very active towards insect species with acidic guts and a typical pattern of cysteine and aspartic proteinase activity, suggesting that the usefulness of commercial proteinaceous inhibitors (possibly depending on the purity) for the classification of insect proteinase activities is questionable. Plant proteins identified as effective inhibitors of insect proteinases are better expressed as recombinant proteinase inhibitor genes in *E.coli* (Michaud *et al.*, 1992; Jongsma *et al.*, 1995). Purified recombinant proteinaceous inhibitors can then be analyzed with confidence for their effectiveness against purified insect proteinases. The gene for proteinase inhibitors active against insect proteinases can subsequently be directly incorporated into transgenic plants to confer host plant resistance to those insects.

CHAPTER 6

PHAGE DISPLAY OF A DOUBLE-HEADED PROTEINASE INHIBITOR: ANALYSIS OF THE BINDING DOMAINS OF POTATO PROTEINASE INHIBITOR II

Potato Proteinase Inhibitor II (PI2) is a serine proteinase inhibitor composed of two domains that are thought to bind independently to proteinases. To determine the activities of each domain separately, various inactive and active domain combinations were constructed by substituting amino acid residues in the active domains by alanines. These derivatives were expressed as soluble protein in *Escherichia coli* and exposed on M13-phages as fusions to gene 3 in a phagemid system for monovalent phage display. Inactivation of both active domains by Ala-residues reduced binding of phages to trypsin and chymotrypsin by 95%. Ten times more phages were bound to proteinases by domain II compared to domain I, while a point mutation [Leu^5→Arg] altered the binding specificity of domain I of PI2-phage from chymotrypsin to trypsin. The mutants were used to show that functional PI2-phages mixed with non-functional PI2-phages could be enriched 323,000-fold after three rounds of panning. Thus, these results open up the possibility to use phage display for the selection of engineered PI2-derivatives with improved binding characteristics towards digestive proteinases of plant pests.

INTRODUCTION

Proteinase Inhibitor II (PI2) was first isolated as a 22-23 kD protein-dimer from potato tubers (Bryant *et al.*, 1976; Pearce *et al.*, 1982) and tomato leaves (Plunkett *et al.*, 1982) and was found to inhibit trypsin, chymotrypsin and subtilisin. The PI2 gene was shown to encode a mature protein of 123 amino acids with two putative active domains [Graham *et al.*, 1985; Keil *et al.*, 1986; Sanchez-Serrano *et al.*, 1986; Thornburg *et al.*, 1987). The 3-dimensional crystal structure of the inhibitor-proteinase complex revealed that a stretch of 10 amino acids ($P_{6,5,4,3,2,1}$-$P'_{1,2,3,4}$; nomenclature of Schechter and Berger (1967), corresponding to residues 57-66 of domain II, is interacting in a β-sheet-like manner with the proteinase catalytic domain (Greenblatt *et al.*, 1989).

PI2 has been suggested to be involved in plant defense against plant feeding organisms (Johnson *et al.*, 1989), but equilibrium dissociation constants of PI2 for trypsin-like gut proteinases of different herbivores have been shown to differ 100-fold (Christeller and Shaw, 1989). At the same time, strongly binding plant PIs have appeared to be more effective resistance factors against plant feeding insects than weaker ones (Burgess *et al.*, 1991; Gatehouse *et al.*, 1993). This suggests that increasing the affinity of PI2 for specific proteinases of target insects might result in PI2 variants with a better potential as resistance factors in transgenic plants. Rational mutagenesis to improve binding domains is mostly hampered by the lack of information on the molecular structure of the specific

inhibitor/proteinase interaction, and, therefore, a random approach is preferable. Phage display has been a successful method for affinity-based selection of protein libraries displayed on the surface of M13 phages for a number of different proteins (Corey *et al.*, 1993; Lowman and Wells, 1993; Marks *et al.*, 1992; McCafferty *et al.*, 1991; Pannekoek *et al.*, 1993; Robertson, 1993; Swimmer *et al.*, 1992). In particular, it was demonstrated for single domain Kunitz inhibitors (Dennis and Lazarus, 1994a,b; Roberts *et al.*, 1992) that variants with dissociation constants in the nanomolar to picomolar range could be selected from engineered libraries of activity mutants by several rounds of "panning" (binding, washing, eluting, and amplifying specific phages) against proteinase bound to a solid support.

We chose to express a cDNA fragment isolated from potato encoding the mature protein of PI2 in a phagemid system for monovalent phage display. In this report the relative contributions of the two inhibitory domains to proteinase binding were investigated by substitution of amino acids in the reactive site by alanine residues. Using these derivatives, we subsequently demonstrated that M13 phages displaying functional PI2 molecules can be efficiently selected from non-functional PI2-phages by consecutive panning rounds.

MATERIALS AND METHODS

E.coli strains

TG1: K12, Δ(lac-pro), supE, *thi*, *hsdD5*/F'*traD*36, *pro*A$^+$B$^+$, *lacI*^{q}, *lacZ*ΔM15
HB2151: K12, *ara*, Δ(*lac-pro*), *thi*/*F'pro*A$^+$B$^+$, *lacI*^{q}ZΔM15

Oligonucleotides

The following PCR-primers (Eurogentec, Seraing, Belgium) were used for mutagenesis. Triplets correspond to amino acid codons. DNA sequences different from the cDNA sequence are in boldface; restriction sites are underlined.
PRIMER1FO: 5'-CGC GCC ATG GCG AAA GCT TGC ACT C(G/T)A GAA TGT GGT AAT CTT GGG TTT GG; sites *Nco*I, *Hin*dIII, *Xho*I/*Xba*I
PRIMER2FO: 5'-CGC GCC ATG GCG AAA GCT TGC GCC GCG GCA TGT GGT AAT CTT GGG TTT GG; sites: *Nco*I, *Hin*dIII, *Sac*II
PRIMER3FO: 5'-C GCA CCG CGG CGC GCA TGC GCT GCA GCT TGC GAT CCA CAT ATT GCC; sites: *Sac*II, *Sph*I, *Pst*I
PRIMER4BA: 5'-GGG TGC GGC CGC TTC CAT TGC AGG GTA CAT ATT TGC; sites: *Not*I
PRIMER5BA: 5'-G CCA CTG CAG TTT GCA TGC TTT TGG TTT TTT TGG G; sites: *Pst*I, *Sph*I

Vector constructions

Vector pB3 was constructed from the phagemid pCANTAB5 (Pharmacia, P-L Biochemicals), by replacing the 1271 bp *Nco*I-*Eco*RI fragment containing the M13 gene 3 fragment by the 1316 bp *Nco*I-*Eco*RI fragment from pHEN1 (Hoogenboom *et al.*, 1991; kindly supplied by Dr. G. Winter from MRC, Cambridge, UK). Consequently, pB3 carries the cmyc-tag of pHEN1 fused to the N-terminus of M13 gene 3, but does not have the *Sph*I site and low phage titers of pHEN1 (Fig. 1). A full length PI2 cDNA clone, p303.51, was selected from

a tuber-specific potato cDNA library using p303 as a probe (Stiekema *et al.*, 1988), and sequenced on an Applied Biosystems 370A nucleotide sequencing apparatus. The part coding for the mature protein (Fig. 2) was amplified by PCR primers to generate the required PI2 variants (Table 1): PI2/PI2[Arg^5]: PRIMER1FO and PRIMER4BA to amplify the part of the cDNA encoding the mature protein. PRIMER1FO was degenerate in one nucleotide resulting in either Leu^5 (PI2) or Arg^5 (PI2[Arg^5]) at the reactive site of domain I. PI2[Ala^{4-6}]: PRIMER2FO and PRIMER5BA to amplify domain I. It was used to replace the *NcoI-SphI* fragment of PI2. PI2[Ala^{61-63}]/PI2[Arg^5,Ala^{61-63}]: PRIMER3FO and PRIMER4BA to amplify the domain II. It was used to replace the *SphI-NotI* fragment of PI2 and PI2[Arg^{62}]. PI2[$Ala^{4-6,61-63}$]: Domain I of PI2[Ala^{4-6}] and domain II of PI2[Ala^{61-63}] were combined at the *SphI* site. All PCR-derived fragments were verified by DNA sequencing.

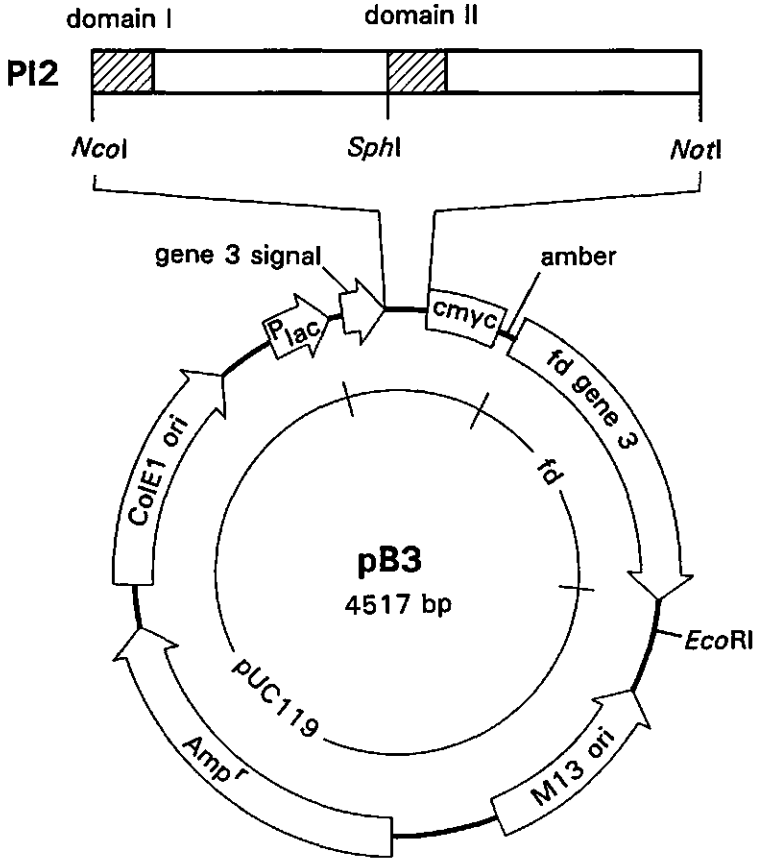

Figure 1. Structure of phagemid vector pB3 used for soluble expression in E.coli and for display of PI2 on the surface of phage

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5'-AAG GCT TGC ACT TTA GAA TGT GGT AAT CTT GGG TTT GGG ATA TGC
1   K   A   C   T   L   E   C   G   N   L   G   F   G   I   C
                    P1  P'1
    CCA CGT TCA GAA GGA AGT CCG GAA AAT CGC ATA TGC ACC AAC TGT
16  P   R   S   E   G   S   P   E   N   R   I   C   T   N   C

    TGT GCA GGT TAT AAA GGT TGC AAT TAT TAT AGT GCA AAT GGG GCT
31  C   A   G   Y   K   G   C   N   Y   Y   S   A   N   G   A

    TTC ATT TGT GAA GGA GAA TCT GAC CCA AAA AAA CCA AAA GCA TGC
46  F   I   C   E   G   E   S   D   P   K   K   P   K   A   C

    CCC CGA AAT TGC GAT CCA CAT ATT GCC TAC TCA AAG TGT CCC CGT
61  P   R   N   C   D   P   H   I   A   Y   S   K   C   P   R
        P1  P'1
    TCA GAA GGA AAA TCG CTA ATT TAT CCC ACC GGA TGT ACC ACA TGC
76  S   E   G   K   S   L   I   Y   P   T   G   C   T   T   C

    TGC ACA GGG TAC AAG GGT TGC TAC TAT TTC GGT AAA AAT GGC AAG
91  C   T   G   Y   K   G   C   Y   Y   F   G   K   N   G   K

    TTT GTA TGT GAA GGA GAG AGT GAT GAG CCC AAG GCA AAT ATG TAC
106 F   V   C   E   G   E   S   D   E   P   K   A   N   M   Y

    CCT GCA ATG TGA
121 P   A   M   -
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Figure 2. cDNA fragment encoding the mature protein of a full length cDNA clone p303.51. P_1-P_1' denotes the putative proteinase cleavage site and centre of the binding domain. Bold lettering indicates the residues that were substituted by alanines to obtain inactive variants. Underlined is the SphI site which was used for subcloning PCR amplified fragments.

Affinity purification and activity assay of soluble PI2

Soluble PI2 was obtained by growing single colonies of *E.coli* strain HB2151 with the various constructs overnight in 10 ml LB-medium, 100 mg/l ampicillin, 1% glucose at 37°C while shaking. 5 ml of the overnight culture was used to inoculate 500 ml LB-medium, 100 mg/l ampicillin, and grown shaking at 25°C to OD_{600} 0.5. Cells were pelleted at 4000×g for 10 min and resuspended in 50 mM NaCl. Cells were respun at 4000×g for 10 min and resuspended in LB-medium, 100 mg/l ampicillin and 1 mM IPTG. Cultures were incubated at 25°C, shaking for 3 hrs. Cells were placed on ice for 20 min, then pelleted at 4000×g for 10 min at 4°C and resuspended in 5 ml ice cold 200 mM sodium borate, pH 8.0, 160 mM NaCl, 1 mM EDTA. Subsequently, cells were pelleted at 4000×g for 10 min at 4°C. The supernatant was heated for 15 min at 85°C and then respun at 20,000×g for 20 min at 4°C. Supernatants were dialyzed overnight against PBS and stored at -20°C for affinity purification.

Affi-T (BioRad) purified anti-cmyc monoclonal antibody (9E10, obtained from Dr. A. Schots, Lab. for Monoclonal Antibodies, Wageningen) was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotech). 3 ml columns were loaded with 1 ml of 9E10-Sepharose 4B slurry and washed extensively with PBS (100 mM sodiumphosphate, 1% NaCl, pH 7.6) under gravity. The PBS-dialyzed sample was run through the column and the column

was washed with 5ml of PBS; 5 ml PBS, 0.5 M NaCl; 5 ml 0.2 M glycine pH 6.0; 5 ml 0.2 M glycine pH 5.0. PI2-cmyc-fusion proteins were eluted with 5 ml glycine buffer, pH2.2. Fractions containing PI2-cmyc fusion protein were neutralized with 3/50 volume of 2 M Tris and concentrated using a 5 kD filter unit (Centrisart I from Sartorius AG, Göttingen).

Table 1. PI2-derivatives constructed to measure the activity and specificity of each domain.

Constructs	Amino acid sequence[a]	
	Domain I pos. 3-7	Domain II pos. 60-64
pB3	none	none
PI2	Cys Thr Leu^{4}Glu Cys	Cys Pro Arg^{4}Asn Cys
PI2[Ala$^{4-6}$]	Cys **Ala Ala Ala** Cys	Cys Pro Arg^{4}Asn Cys
PI2[Ala$^{61-63}$]	Cys Thr Leu^{4}Glu Cys	Cys **Ala Ala Ala** Cys
PI2[Ala$^{4-6,61-63}$]	Cys **Ala Ala Ala** Cys	Cys **Ala Ala Ala** Cys
PI2[Arg^{5}]	Cys Thr Arg^{4}Glu Cys	Cys Pro Arg^{4}Asn Cys
PI2[Arg^{5},Ala$^{61-63}$]	Cys Thr Arg^{4}Glu Cys	Cys **Ala Ala Ala** Cys

[a] The arrows indicate the peptide bond of the active site (P_1-P'_1). Bold lettering is used for mutant amino acid residues.

Activities of the PI2-fractions were very low and were, therefore, detected in a an assay modified from Jongsma *et al.* (1993). Into petri-dishes (9 cm diameter) 6 ml melted agar solution (Jongsma *et al.*, 1993) was poured containing 5 nM chymotrypsin or 10 nM trypsin. After solidification 10 μl inhibitor solutions of even concentration (estimated from Coomassie Brilliant Blue stained gels) were applied on top of the agar. After 4 hours the plates were developed as described (Jongsma *et al.*, 1993) using the substrate acetylphenylalanine-β-naphthylester. The β-naphthol product of hydrolysis immediately complexed in a diazocouplings reaction to form an insoluble coloured precipitate. Clear zones represented inhibited proteinase.

Isolation and purification of PI2-phages

All PI2-variants cloned in pB3 in *E.coli* strain TG1 (Sambrook *et al.*, 1989) were plated on MM-plates (100 mg/l ampicillin, 0.2% glucose, and 1 mM thiamine-HCl). One ml of SB-medium (Barbas *et al.*, 1991) with 100 mg/l ampicillin, 2% glucose was inoculated and grown for 4 hours at 37^{0}C. Subsequently, 0.1 ml was diluted 10 times with culture medium and 2.5 $\times$ 10^{9} VCSM13 helperphage were added. The culture was incubated at 37^{0}C for 30 min in a waterbath, subsequently for again 30 min in a shaker at 300 rpm. Cells were pelleted for 2 min at 6500$\times$g and used to inoculate 10 ml SB-medium (100 mg/ml ampicillin, 70 mg/ml kanamycin) in 50 ml flasks. Cultures were incubated overnight at 28^{0}C and 300 rpm.

Cells were removed by centrifugation at 12,000$\times$g for 20 min. Phages were precipitated from the supernatant for 1 hour on ice after adding 0.25 volumes of 2 M NaCl,

16% PEG-8000. Phageparticles were pelleted at 12,000×g for 20 min and resuspended in 1 ml PBS. Particles were precipitated again by the same procedure, but resuspended in 0.4 ml 0.1 N HCl adjusted to pH2.2 with glycine, containing 1 mg/ml fatty acid free BSA and filtered using a 0.45 μm filter according to Corey *et al.* (1993). It is possible that during the production of phages *E. coli* proteinases will be bound by PI2-phages. PI2-phages that are complexed to proteinases in solution will show reduced binding to immobilized proteinases. In acid dissociation of the inhibitor-proteinase complex is achieved allowing for the removal of most bound *E.coli* proteinases, other proteins and phage debris by washing the phage suspension with 10-15 volumes of the low pH buffer using a 300 kDa filter unit (Amicon).

Binding and elution of PI2-phages

Microtiterplates (TC, Greiner) were coated with bovine trypsin (type III, Sigma), bovine α-chymotrypsin (type II, Sigma) or BSA. In a protein solution of 0.2 mg/ml in 50 mM Tris-Cl, pH7.8, 0.01 volumes of 25% glutaraldehyde was mixed and wells were filled with 0.2 ml of this solution. After 45 min the wells were emptied and washed 3 times with PBS. The quantity of bound active enzyme was measured enzymatically to be about 200 ng/well. Wells were incubated with 0.2 ml blocking buffer (2% (w/v) nonfat dry milk in PBS) for 60 min and washed again 3 times with PBS. A mix of 0.1 ml blocking buffer and 0.1 ml 0.5-1×10^{10} cfu of acid-purified and neutralized phages was prepared 15 min in advance of addition to a microtiter well. The mix was added to the well for 1 hour. Non-specifically bound phages were removed by washing 5 times with PBS, 10 times with PBS, containing 0.5% Tween-20. Subsequently, wells were washed once for 5 min with 50 mM sodiumcitrate buffers (containing 1 mg/ml BSA) of decreasing pH (pH 6-5-4-3) to further reduce the background of weakly binding PI2[$Ala^{4-6,61-63}$]-phages. At this point bound phages were either detected by ELISA or eluted with 100 μl glycine-buffer pH 2.2 for 30 min. Eluted phages were neutralized with 6 μl 2 M Tris. Titers were determined by incubating 100 μl log-phase TG1 cells with 10 μl phage eluate dilutions for 20 min at 37°C and plating on LB-plates containing 100 mg/l ampicillin.

Solid amplification of eluted phages after panning

Two ml TG1 cells in log-phase were infected with 50 μl of the eluate for 30 min at 37 °C and subsequently incubated for 30 min at 37°C with shaking. Cells were spun down and plated on a SOBAG plate (SOB-medium (Sambrook *et al.*, 1989) without potassium chloride containing 15 g/l agar, 2% glucose, 100 mg/l ampicillin). Cells were grown overnight at 28°C. The confluent cell layer was collected in 5 ml SB-medium, diluted to OD_{600} 0.3 in 10 ml SB-medium, 100 mg/l ampicillin, 2% glucose. Cells were grown shaking at 37°C to OD_{600} 0.7. At that point 2.5 × 10^9/ml VCSM13 helper phages were added. The procedure from then on was identical to the PI2-phage isolation protocol above.

RESULTS

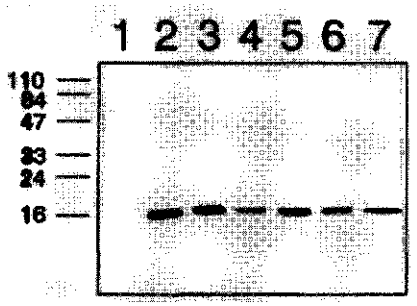
Construction of active domain derivatives

In order to determine the proteinase inhibitor activity of each of the two active domains separately, two sets of mutants were made. In the first set inactive domains were created by

substituting three amino acids in the active domains of PI2 (Thr^4-Leu^5-Glu^6 and Pro^{61}-Arg^{62}-Asn^{63}), that were known to be involved in the interaction with the proteinase catalytic domain, by Ala-triplets to reveal the activity of the complementary domain. This resulted in three derivatives of PI2 listed in table 1: (i) domain I inactive (PI2[Ala$^{4-6}$]); (ii) domain II inactive (PI2[Ala$^{61-63}$]); (iii) both domain I and II inactive (PI2[Ala$^{4-6,61-63}$]). In the second set the active site residue P_1 which is considered to determine the specificity towards chymotrypsin (P_1=Leu, Phe) and trypsin (P_1=Arg) (Hass *et al.*, 1982; Richardson, 1979), was changed for domain I from Leu^5 into Arg^5. These two additional derivatives are also listed in table 1: (i) domain I trypsin specific (PI2[Arg^5]); (ii) domain I trypsin specific, domain II inactive (PI2[Arg^5,Ala$^{61-63}$]). To assess the activity of these derivatives they were expressed in *E. coli*.

Characterization of soluble PI2-cmyc fusion proteins expressed in E.coli

All PI2-derivatives were expressed in *E. coli* as soluble proteins fused to the 18 amino acid long cmyc-tag to allow easy purification by affinity chromatography using monoclonal antibody 9E10. Periplasmic fractions of *E. coli* cells expressing the PI2-cmyc fusion proteins



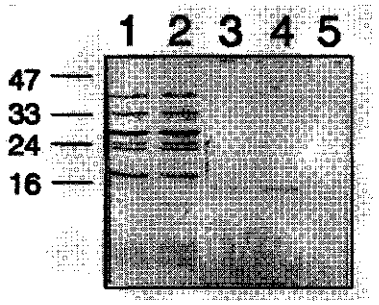


Figure 3. A: Western blot analysis of PI2-cmyc fusion peptides. The periplasmic protein fraction which was used for affinity purification was subjected to electrophoresis on 15% polyacrylamide/SDS gels. Resolved proteins were electroblotted onto nitrocellulose and probed with anti-cmyc monoclonal antibody. Rabbit anti-mouse IgG conjugated to alkaline phosphatase (Sigma) was used for staining. Lane 1, control cells transformed with pB3; Lanes 2-7: PI2[Ala$^{4-6,61-63}$], PI2, PI2[Arg^5], PI2[Ala$^{4-6}$], PI2[Ala$^{61-63}$], PI2[Arg^5,Ala$^{61-63}$].

B. Coomassie brilliant blue stained gel of affinity purified PI2-cmyc fusion peptide. Periplasmic protein was applied on a 9E10-Sepharose 4B affinity column. Bound fusion peptide was eluted with acid as described under Materials and Methods. The input protein (lane 1), non-bound fraction (lane 2), and subsequent fractions eluted with pH 2.2 buffer (lanes 3,4,5) were separated on 15% polyacrylamide/SDS gels and stained.

were isolated and analyzed by western blotting using 9E10. All six PI2-derivatives produced a fusion protein of M_r 16 kD (Fig 3A) close to the calculated MW of 15.1 kD. No breakdown products could be detected for any of the six constructs. The PI2 fusion protein could be purified in a one step procedure using a 9E10-Sepharose 4B column (Fig. 3B).

Purified proteins were obtained in small amounts and, therefore, analyzed using a qualitative assay for the presence or absence of inhibitory activity towards trypsin and chymotrypsin. The assay was based on the diffusion of proteinase inhibitors into a thin agar

layer containing trypsin or chymotrypsin (Fig. 4). It was found that PI2 and PI2-derivatives with domain II active (PI2[Arg^5]), but domain I inactive (PI2[Ala^{4-6}]) inhibited both trypsin and chymotrypsin. In contrast, derivatives with domain II inactive (PI2[$Ala^{4-6,61-63}$]), but domain I active (PI2[Ala^{61-63}], PI2[Arg^5,Ala^{61-63}]) were found to be poor inhibitors of both enzymes. These results indicated that domain II of PI2 is apparently specific for both trypsin and chymotrypsin, while domain I displayed no detectable activity at the given enzyme concentration.

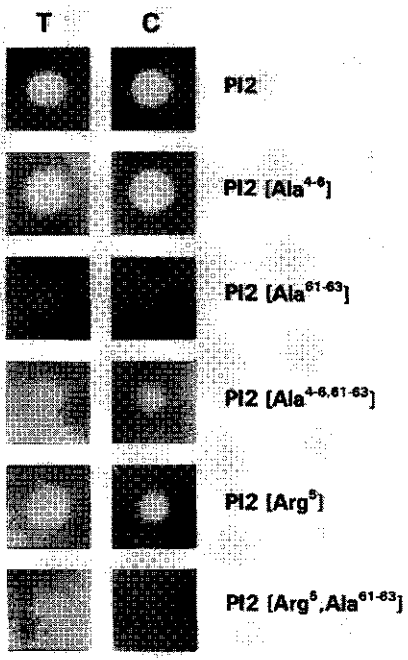


Figure 4. Qualitative assay of proteinase inhibitor activities of affinity-purified, soluble PI2-cmyc fusion proteins relative to chymotrypsin (C) and trypsin (T) as indicated by clear zones in a coloured background. Inactive mutants weakly inhibit proteinase activity causing some background inhibition.

Binding and elution of PI2 derivatives displayed on phages

PI2-derivatives were produced in *E.coli* as translational fusions to gene 3 by switching to *E. coli* strain TG1 which suppressed the amber stopcodon between PI2-cmyc and gene 3 (Fig. 1). Co-infection with helperphage resulted in the incorporation of the PI2-cmyc-gene3 fusion protein in the coat protein of secreted phages. Western blot analysis of phage protein showed fusion protein of the expected molecular weight (M_r 81kD) as the predominant band using either anti-cmyc monoclonal antibody (Fig. 5, lanes 2-7) or antiserum raised against PI2 (not shown). The cmyc-gene 3 protein encoded by pB3 migrated as a 67 kD protein (Fig.5, lane 1).

To examine the binding of PI2-phages to trypsin and chymotrypsin coated to microtiterplate wells, phage binding was quantitated by ELISA using antiserum directed against coat proteins of the phage. PI2-phages bound strongly to proteinases as evidenced by

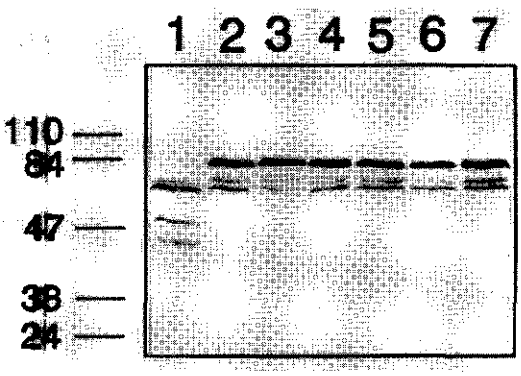


Figure 5. Western blot analysis of recombinant phage particles carrying PI2-cmyc-gene 3 fusion proteins. Equivalent numbers of phage were subjected to electrophoresis on 10% polyacrylamide/SDS gels. Resolved proteins were electroblotted onto nitrocellulose and probed by using 9E10 mouse anti-cmyc monoclonal antibody. Cmyc-gene 3 fusion proteins (pB3) migrate at 67 kD (lane 1), while PI2-cmyc-gene 3 fusion proteins migrate at 81 kD (Lanes 2-7: PI2[$Ala^{4-6,61-63}$], PI2, PI2[Arg^{5}], PI2[Ala^{61-63}], PI2[Ala^{61-63}], PI2[Arg^{5},Ala^{61-63}]).

the high ELISA-readings relative to vector-phage (pB3) (Table 2). This showed that PI2 was exposed on the phage tip as a functional protein. Substitution by alanine residues in both domains resulted in PI2-fusion protein (PI2[$Ala^{4-6,61-63}$]) with strongly reduced affinity for proteinases, showing that binding was specifically mediated by these domains. Complete inactivation of activity was not obtained, however, as evidenced by the above background ELISA readings for PI2[$Ala^{4-6,61-63}$] relative to pB3 (Table 2). Apparently, also other amino acids of the active domains contributed to proteinase binding. The binding of phages of which only the first domain was inactivated (PI2[Ala^{4-6}]) did not significantly differ from the PI2-phages confirming the results with soluble PI2 that domain II accounts for most of the activity of PI2 to both trypsin as well as to chymotrypsin. Indeed, when instead of domain I only domain II was inactivated (PI2[Ala^{61-63}], PI2[Arg^{5},Ala^{61-63}]), binding of the phages was strongly reduced. The residual binding of these phages mediated by domain I only (PI2[Ala^{61-63}]) is specific, since changing P_1-residue Leu^{5} into Arg (PI2[Arg^{5},Ala^{61-63}]) shifted the binding preference as expected from chymotrypsin to trypsin.

Determination of the eluted phage titers at pH 2.2 in a parallel experiment confirmed the results obtained from ELISA readings. Table 2 shows that relative to inactive PI2-phage (PI2[$Ala^{4-6,61-63}$]) domain I resulted in 2-fold (PI2[Ala^{61-63}] on chymotrypsin) or 9-fold (PI2[Arg^{5},Ala^{61-63}] on trypsin) higher titers, whereas domain II (PI2[Ala^{4-6}]) resulted in around 100-fold higher titers on trypsin and somewhat lower factors on chymotrypsin. In any one experiment eluted phage titers could differ by a factor of two, so that only differences of an order of magnitude were significant. Thus, phage titer ratios obtained using pure phage stocks predicted, that in a mixture of functional (e.g. PI2[Ala^{4-6}]) and non-functional (PI2[$Ala^{4-6,61-63}$]) PI2-phages enrichment factors of two orders of magnitude should be observed.

Panning a mixture of functional and non-functional PI2-phages

PI2-phages active only in domain II (PI2[Ala^{4-6}]) were mixed with PI2-phages inactive in both domains (PI2[$Ala^{4-6,61-63}$]) in a ratio of 1:100, 1:1000 and 1:10,000 to determine the efficiency at which rare functional PI2-phages could be purified from the mixture. Phage mixtures were enriched for functional PI2-phages by three consecutive rounds of panning against trypsin. After each round phage titers were determined to obtain an indirect indication of the ratio of functional to non-functional PI2-phages. After the second round higher titers

Table 2. Binding and elution of PI2-phage derivatives adsorbed to immobilized trypsin or chymotrypsin.

Immobilized proteinase	Construct	ELISA readings (OD_{410})[b]	Eluted phage titers[c]	Titer ratio relative to pB3[d]	Titer ratio relative to PI2[$Ala^{4-6,61-63}$][d]
Trypsin[a]	pB3	0.007	6,400	1	-
	PI2	1.732	1,700,000	264	89
	PI2[Ala^{4-6}]	1.577	2,100,000	326	111
	PI2[Ala^{61-63}]	0.110	37,000	6	2
	PI2[$Ala^{4-6,61-63}$]	0.089	19,000	3	1
	PI2[Arg^{5}]	1.390	2,100,000	326	111
	PI2[Arg^{5},Ala^{61-63}]	0.318	180,000	28	9
Chymotrypsin[a]	pB3	0.000	2,700	1	-
	PI2	1.063	1,000,000	370	25
	PI2[Ala^{4-6}]	1.184	1,200,000	444	31
	PI2[Ala^{61-63}]	0.139	67,000	25	2
	PI2[$Ala^{4-6,61-63}$]	0.076	39,000	14	1
	PI2[Arg^{5}]	1.115	3,800,000	1407	97
	PI2[Arg^{5},Ala^{61-63}]	0.079	38,000	14	1

[a] 10^{10} cfu were added per well of microtiterplates coated with trypsin or chymotrypsin as described in Materials and Methods.

[b] Coat protein of bound phages was detected according to the manufacturers instructions after the final wash step at pH3 using horseradish peroxidase conjugated to sheep anti-M13 IgG (Pharmacia, P-L Biochemicals). The values are means of duplicate determinations after substraction of the substrate blank.

[c] Phages were eluted at pH2.2 and titers were determined in duplicate as described. Phage titers varied as much as two-fold between duplicates.

[d] The titer ratios relative to pB3 or PI2[$Ala^{4-6,61-63}$] are calculated on the basis of the fraction of input phage that has been recovered after elution.

compared to the control (PI2[$Ala^{4-6,61-63}$]) were observed for the first time (Table 3). After the third round all three mixtures clearly had higher titers, suggesting that the majority of phages in all three mixtures were then functional PI2[Ala^{4-6}] phages.

To assess this directly, phage mixtures were probed for the presence of functional PI2-phages using a ^{32}P end-labeled oligonucleotide in a colony filter hybridization assay. Table 3 shows that after the third round of enrichment the 10,000-fold diluted PI2[Ala^{4-6}] phage represented 97% of the phage population indicating 323,000-fold enrichment. A low percentage (3-7%) of phages not hybridizing to the functional domain probe remained present also in the phage mixtures of low dilution. This suggested that there is an upper limit of about 95% for functional purity.

Table 3. The enrichment process of functional PI2[Ala$^{4-6}$]-phages mixed with non-binding PI2[Ala$^{4-6,61-63}$] phages in three successive rounds of panning.

phage mixture		control PI2[Ala$^{4-6,61-63}$]	control PI2[Ala$^{4-6}$]	1:100[a]	1:1000[a]	1:10,000[a]
eluted phage titer[b]	round 1	40,600	450,000	38,400	42,400	57,600
	round 2	34,400	2,920,000	540,000	81,600	32,400
	round 3	120,000	1,910,000	2,680,000	2,490,000	950,000
percentage PI2[Ala$^{4-6}$] phages[c]	round 1	0	100	8	2	<1
	round 2	0	100	86	49	13
	round 3	0	100	97	93	97

[a] Starting ratios of functional PI2[Ala$^{4-6}$] phages relative to non-functional PI2[Ala$^{4-6,61-63}$] phages

[b] The starting phage titers were 2-10 × 10^9

[c] Percentage PI2[Ala$^{4-6}$] phages among the total number after elution and amplification of phages. These data were obtained by colony filter hybridization of 100 cfu's with a PI2[Ala$^{4-6}$]-specific oligonucleotide probe 5'-TGCCCCCGAAATTG-3' which was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Sambrook *et al.*, 1989).

DISCUSSION

In this paper results are presented which show the relative activities of the two inhibitory domains present on PI2, both as soluble proteins and as part of the protein coat of M13-phages. Binding to proteinases was investigated by selectively disrupting the activity of these domains, separately or simultaneously, by substituting a region of three amino acids by alanine residues. This region, flanked by two cysteine residues, was known to be involved in a β-sheet-like interaction with the proteinase catalytic domain without an obvious role for folding of the protein itself (Greenblatt *et al.*, 1989).

*Wt*PI2, both as a soluble protein and displayed on the surface of phage, was found to bind specifically to trypsin and chymotrypsin. This binding was mediated by the active domains, as substitution by alanine residues in both domains (PI2[Ala$^{4-6,61-63}$]) led to a binding reduction of 95%. The observed 5% residual binding of this derivative on phages indicated that more than the three substituted amino acids in the active domain are involved in proteinase binding. Substitution by alanines of only domain I did not reduce binding of PI2-phages and soluble protein compared to *wildtype*. This demonstrated, firstly, that such mutations do not disrupt the function of the protein, and, secondly, that most likely domain I was not very active in comparison to domain II. Indeed, inactivation of only domain II strongly reduced binding, confirming that domain II was responsible for most of the activity against trypsin as well as chymotrypsin. The dual specificity of domain II for trypsin and chymotrypsin was unexpected, because highly homologous single domain PI2 proteins from eggplant and potato, lacking 24 N-terminal and 47 C-terminal residues, but with Arg^{62} at the P_1-position, were found to be specific for trypsin only (Hass *et al.*, 1982; Richardson, 1979). In contrast, the P_1-residue of domain I did determine the specificity. Phages with Leu as P_1-residue in domain I (inactive domain II) bound to chymotrypsin and not to trypsin and vice

versa with Arg as P_1-residue. The low activity of domain I towards bovine proteinases both as a soluble protein and on the surface of phages may be an intrinsic property of the inhibitor. A stretch of chain of 10 amino acids of domain II were shown to be involved in the binding to *Streptomyces griseus* proteinase B (Greenblatt *et al.*, 1989). Six out of those ten residues differ between domains I and II, and the difference in activity between both domains may, thus, point to specialization of domains with respect to different gut proteinases (Christeller and Shaw, 1989).

We demonstrated that functional PI2-phages mixed in a ratio of 1:10,000 with non-functional PI2-phages could be enriched 323,000-fold in three rounds of panning. These PI2-phages were different by only three amino acids demonstrating the powerful functional selectivity of the method. The observation that the $Leu^5 \rightarrow Arg$ substitution in domain I altered specific binding from chymotrypsin to trypsin suggested that even a single amino acid residue difference could be selected for. The high enrichment factor indicates that engineered libraries of active domain variants can be much larger than 10,000, especially, if an additional round of panning is performed. It was apparent from the mixtures with smaller ratios of functional to non-functional phages that a small percentage of phages without the functional domain remained present. We did not investigate further whether these phages represented the original non-functional PI2-phages, or, in fact, contained spontaneous deletions allowing phages to replicate more rapidly. For the practical purpose of panning engineered libraries of domain II the percentage of non-functional PI2-phages (3-7%) remains low enough to present no major problem in the analysis of panned libraries.

Many plant proteinase inhibitors are multidomain proteins with two (potato/tomato PI2), five (tobacco PI2 (Atkinson *et al.*, 1993)), or even eight repeated units (multicystatin (Waldron *et al.*, 1993)). They offer advantages when compared to single domain inhibitors to simultaneously control different proteinases of one or more insect pests. The phage display system presented here provides an attractive method to selectively improve PI2 domains against target insect proteinases by engineering a library of domain variants and inactivating the complementary domain. The specific improvement and subsequent rational combination of PI2 domains for expression in plants will allow a more effective use to be made of the inhibitor in engineered plant protection against insect pests (Hilder *et al.*, 1987; Johnson *et al.*, 1989; McManus *et al.*, 1994).

CHAPTER 7

GENERAL DISCUSSION

The aim of this study was to investigate the role of endogenous induced proteinase inhibitors (PIs), and the potential of transgenic PIs, in the defense of plants against insects. Below, the results are discussed in sections dealing with the responses occurring in the plant and the insect, respectively, when lepidopteran larvae are feeding on solanaceous plants. Subsequently, these results are taken to defend the proposition that the success of PIs in plant resistance against insects will depend on the application of PIs inhibiting the induced proteinases that are insensitive to host plant PIs. It is argued that the isolation of PIs with such novel properties can be achieved by the phage display method.

The plant's response to attack by insects

In the first part of the thesis (Chapters 2 and 3) it is shown that insect feeding, wounding and virus infection, induce PI activity in the wounded or infected leaves of mature tobacco and tomato plants, but not in non-wounded or non-infected leaves. Originally, work with tomato and tobacco seedlings had created the idea that PIs were always induced in both wounded and non-wounded tissues of these plants (Nelson *et al.*, 1983; Pearce *et al.*, 1993). However, Wolfson and Murdock (1990) demonstrated that the ability of tomato plants to induce PIs after wounding gradually disappeared in non-wounded leaves during maturation of the plant. Our results demonstrate that *local* PI induction is *not* affected by maturation, so that herbivorous insects are still confronted with high induced PI levels. In addition, we have shown that local induction of PI activity is, nevertheless, under the influence of a systemic signal, which amplifies the PI response when several leaves are wounded at the same time. These two results suggest that, when plants mature, wounding results in a quiet alarm of the unwounded plant parts, while near the wound PIs are strongly induced, independent of plant age, to deter the herbivore.

Different explanations can be proposed for the disappearance of systemic PI induction in maturing tobacco and tomato plants. Mechanistic explanations argue that during plant development young leaves initially serve as sinks of phloem-transported assimilates, but then rapidly become sources of these assimilates. Some phloem-transported signal molecules like systemin (Pearce *et al.*, 1991) are very likely also mainly directed to sinks of assimilates like growing meristems. Thus, the distribution of systemically induced PIs within the plant depends on plant age (Wolfson and Murdock, 1990), leaf position, and the changing sink-source relationships between leaves (Davis *et al.*, 1991). Furthermore, our finding that wounding two leaves results in a two-fold stronger local PI induction compared to wounding either leaf alone also allows a more functional explanation. It can be argued, that, when damage by insects remains restricted to a single leaf among many others, it is an overreaction to respond with the induction of PI activity in all leaves. In such cases a quiet alarm in the unwounded leaves, which allows the leaves to respond more strongly when they are attacked

as well, is much more appropriate (Fig. 1).

After it was found that PI activity was strongly induced by insect feeding it seemed pointless to introduce additional foreign PI genes into tobacco. On the other hand, the successes with transgenic tobacco plants expressing PIs reported in the literature, suggested that *constitutive* PI levels might provide a more effective defense than an inducible response (Hilder *et al.*, 1987; Johnson *et al.*, 1989; McManus *et al.*, 1994). In chapter 4 it is shown that the time gained by constitutive versus inducible PI gene expression is relatively small. Locally, effective levels of PI activity were already reached after 24 hours of insect feeding, which indicated that the reported success with transgenic plants should mainly be attributed to greater specific inhibiting activity of transgenic PIs relative to induced endogenous PI activities and not so much to high constitutive PI expression levels.

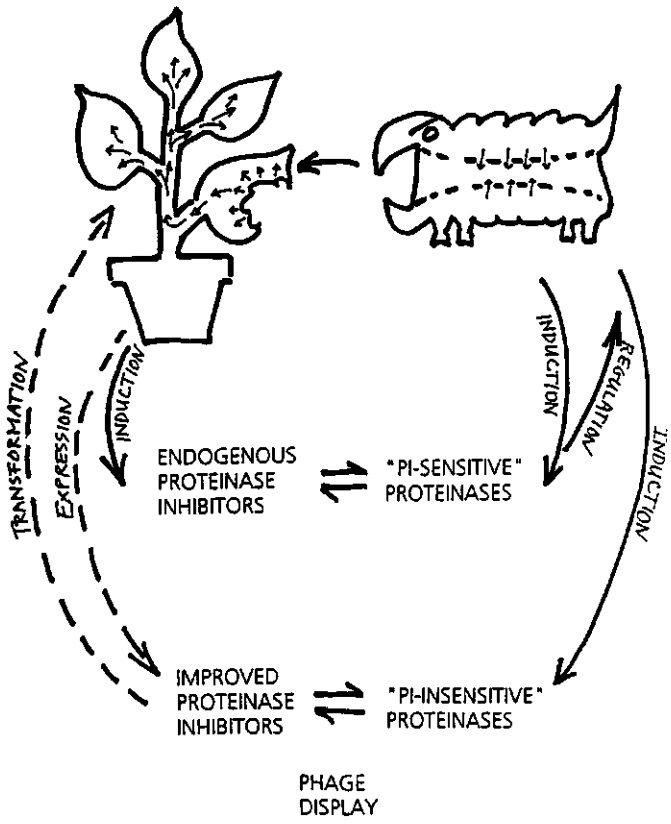


Figure 1. Schematic diagram of the events that occur when *S. exigua* larvae feed on tobacco leaves. The leaf damage caused by the larvae results in the local (mature plants) and systemic (young plants) induction of high levels of proteinase inhibitors. Ingestion of induced leaves results in the inhibition of digestive proteinases in the larval midgut. In response to the decrease of gut proteinase activity, proteinases insensitive to the plant PIs are induced. Phage display is expected to facilitate the isolation of PIs that are active against proteinases that are presently not inhibitable by potato PI2 or endogenous tobacco PIs.

The insect's response to defense proteins of plants

In chapter 4 results are presented, that demonstrate that *S. exigua* larvae, reared on tobacco leaves containing transgenic PI2 or endogenous tobacco PIs, loose proteinase activity in their guts due to inhibition, but largely compensate for that loss by increasing the level of PI2-insensitive activity 2.5-3-fold above normal levels. The switch to other proteinases in response to PIs allows the larvae to grow normally, even though proteinase activity in gut extracts in the presence of PIs was decreased to 71% of the control. This mechanism of adaptation, that makes the insects resistant to plant PIs, has not been described before, and may explain why also many other insects are relatively insensitive to the PI-mediated defense of plants.

This mechanism contrasts the current opinion in the literature on the action and effects of PIs. Broadway and Duffey observed in 1986 that PI2 did not reduce proteolytic activity in the guts of *S. exigua* larvae, even though growth was reduced to some extent. They explained these observations by assuming that the insects responded to PIs by synthesizing digestive proteinases at concentrations exceeding the PI concentrations in plants, so that a surplus of uninhibited activity would be available for protein digestion in the gut. It was claimed that this hyperproduction of proteinases depleted rare essential amino acids to the extent that growth was inhibited. This hypothesis has frequently been cited in the literature (e.g. Burgess *et al.*, 1991), but was never based on direct evidence. The results described in chapter 4 prove that the proteolytic activity measured by Broadway and Duffey in *S. exigua* larvae can be attributed to completely different proteinases that are insensitive to PI2. We have observed a small induction of proteinase synthesis, but a relatively strong decrease in the level of protein digestion, which leads us to propose that growth reduction of *S. exigua* larvae (as observed by Broadway and Duffey, 1986) is caused by reduced digestion of plant protein and not by hyperproduction of insect proteinases.

In this thesis adaptation to plant proteinase inhibitors is only demonstrated for a lepidopteran insect, *S. exigua*, which is a generalist herbivore on a wide variety of plant species, including both dicotyledonous and monocotyledonous plants. From a polyphagous insect adaptive mechanisms of this kind can be expected as it must be capable of responding to a large array plant defense compounds, but it is clear that this flexibility is limited as the induced synthesis of PI-insensitive proteinases does not fully restore proteinase activity to its original level. From a monophagous insect, specializing on a single host plant a more effective form of adaptation would be expected either involving full restoration of the original proteinase activity by PI-insensitive proteinases, or even better, full insensitivity of the gut proteinases to host plant PIs independent of exposure to host plant PIs. Nevertheless, very similar results were also found for Colorado potato beetle (CPB) larvae (Bolter and Jongsma, in press). This coleopteran insect is considered a specialist on potato and some related solanaceous plants. When reared on potato plants containing high levels of serine, cysteine and aspartic proteinase inhibitors induced by methyl jasmonate, growth of CPB larvae was not affected by papain inhibitor levels as high as 4% of total soluble leaf protein. It was shown that cysteine and aspartic proteinases, that were insensitive to potato PIs, were induced two-fold in the CPB larvae, and compensated the loss of proteolytic activity due to PIs up to 58% of the control. Even though this reduced level of activity was sufficient to maintain normal growth negative effects on mortality and fertility later on in development may still occur. The limited degree of adaptation may indicate that CPB's under natural conditions do not encounter the high PI levels that were induced by methyl jasmonate. This

possibility is supported by the finding that cysteine proteinase inhibitors in potato are relatively poorly induced by wounding (C.J. Bolter, personal communication), so that the selection pressure for PI-insensitive gut proteinases remains low.

In combination, the results demonstrate that different insect species of different insect orders (lepidopterans and coleopterans) using different classes of proteinases for protein digestion (serine and cysteine or cysteine and aspartic proteinases) adapt to plant PIs by inducing PI-insensitive proteinases, suggesting that this mechanism is general among insects independent of which class of proteinase is used for protein digestion. Remarkably, this type of adaptation has also been observed in vertebrates, like rats and humans, which use plants for food, suggesting that such adaptation to plant PIs is not restricted to insects, but is common among all herbivores (Holm *et al.*, 1988a/b, 1991). The success of this resistance mechanism of herbivores against defense proteins of plants depends on the availability of genes in the gene pool encoding proteinases that are not susceptible to plant PIs. The number of such genes is expected to be small. By expressing in host plants transgenic PIs active against the proteinases of insects, which are not susceptible to the naturally occurring PIs, these plants may become resistant. Such PIs may be sought in unrelated plant species, insects, or other organisms. We propose to engineer the desired specificities into plant PI genes, that are already available, and to select them by phage display. The potential of this approach is discussed below.

Proteinase inhibitors as potential resistance factors against insects

The potential of plant PIs as useful resistance factors against insects depends on our ability to isolate plant PI genes encoding PIs, that are active against insect gut proteinases, which are not inhibited by the PIs of a certain host plant. The classical approach to achieve this would first require the identification and purification of the inhibitor, and then the isolation of the corresponding gene. This procedure would need to be carried out for various PIs from a wide range of plants, and moreover would have to be repeated for every different insect proteinase (Christeller and Shaw, 1989). Apart from the practical pitfalls of such an approach, the most important drawback is the uncertainty, whether the desired inhibitor of these proteinases will actually be found in plants. Proteinase inhibitors can be grouped into a limited number of gene families (Chapter 1: Table 1). The region interacting with the proteinase and determining the specificity is mostly well known, and in most cases these regions are (hyper)variable (Hill and Hastie, 1987). This is illustrated for the active domain of PI2 of different solanaceous plants in Figure 2. The variability of the active domain suggests that specificities towards various proteinases of insects and animals evolved. However, as each plant genome contains an estimated 100 to 200 different PI genes, the available variation in specificities is fairly small, and the desired specifity may not be found.

There is no *a priori* reason to look for active PIs within the plant kingdom only. Many excellent inhibitors of gut proteinases of vertebrates have been isolated from vertebrate tissues. Recently, PIs of vertebrate and insect origin were found to strongly reduce growth and survival of insects, if mixed in artificial diets or expressed in transgenic plants (Czapla, 1994; Thomas *et al.*, 1994). The mode of action of insect PIs in insect guts may be altogether different from plant PIs, however. Iwai *et al.* (1988), for example, demonstrated that a member of the pancreatic secretory trypsin inhibitor (PSTI) family regulates the level of proteinase activity in guts of rats by binding a receptor present on gut epithelial cells. Receptor-binding signals the increased synthesis of proteinases in the pancreas. The effects

of high dietary levels of this inhibitor, if any, are then expected at the level of proteinase gene expression, and less so at the level of proteinase inhibition. Thus, while insects appear to be interesting sources of active PIs, their effects may not correlate with their ability to reduce protein digestion in the gut, and in that respect these inhibitors may not complement the action of hostplant PIs as intended in this thesis.

To avoid the many pitfalls and complications which may arise in isolating naturally occurring PIs and their corresponding genes, we propose a different method of isolating PIs with desired specificities. Gene technology allows the artificial generation of much greater variation in the PI binding domains than found in nature. This is achieved by synthesizing an oligonucleotide with a portion of random sequence, which corresponds to the PI binding domain. If properly designed, this will result in a mixture of oligonucleotides, that can subsequently be used in a polymerase chain reaction (PCR) to generate a mixture (library) of PI gene fragments. If 5-6 amino acids are randomized in the binding domain, a set of 10^7-10^9 different genes is generated. The selection of an inhibitor that is active towards a certain insect proteinase is accomplished by phage display. Phage display is the display of proteins on the surface of phage particles by fusion of a coat protein with a protein of interest. We have demonstrated the potential of phage display using a phagemid vector, in which the PI gene is fused to a minor coat protein (gene 3 protein) of the filamentous *E.coli* phage M13 under the control of the LacZ promoter. After first transforming *E.coli* cells with the phagemid gene library, subsequent infection with helper phage results in the production of phage particles that contain the different PI genes (genotype), and display the different PI protein-variants fused to a minor coat protein on the phage surface (phenotype). Only those phage particles, that possess a domain with affinity for an insect proteinase, are enriched by affinity chromatography. The stringent washing conditions automatically select for the best binders, and the bound phages can be eluted and used for infecting *E.coli* to identify the genes which demonstrate affinity for the target proteinase. In comparison to the classical approach phage display is a more flexible method, because libraries can be re-used to find PIs with other properties, and a positive result is more certain as the method can also generate high affinities and novel specificities not found in nature (Roberts *et al.*, 1992; Dennis and Lazarus, 1994a,b). In addition, an enormous amount of time can be saved. In the phage display procedure it is a matter of weeks to isolate active PI genes from a phage displayed library, whereas in the classical approach it can take years to isolate the right gene.

In chapter 6 it was demonstrated that PI2 can be displayed on the surface of the phage as a functional protein. Phages carrying functional PI2 were enriched 320,000-fold against trypsin in three rounds of panning. This suggests that phage display can be used to select inhibitors against specific proteinases. If it would be possible to make large libraries of engineered variants of PI2, to display them on phages, and to select clones that inhibit purified insect proteinases, the identification of specific PIs would become much more efficient. In recent unpublished work a library of 8000 different PI2 proteins was constructed and selected against bovine chymotrypsin and trypsin. After two rounds of selection binding PI2 variants were selected. This proved the viability of the method with a real library and has opened the way to select the library against insect proteinases. The purification and characterization of *S. exigua* proteinases has been described in chapter 5, and resulted in the identification of a proteinase which is not inhibited by tobacco PIs (unpublished result). Thus, it is now possible to try to select specific inhibitors against *S. exigua* proteinases. Hopefully in the future specific inhibitors will provide a more effective defense against larval attack by complementing host plant PIs in transgenic tobacco (Fig. 1).

A

```
                                                          active domain
                                                               P1P1'
                                                                ||
Dom-2; pot-1    NRICTNCCAGYKGCNYYSANGAFICEGESDPKK|PKACPRNCDP|HIAYSKCPRSEGKSLIY

pot-1.1     1                                     ---TLE-GN|.LGFGI------SPE
pot-2.1     1                                     ---TLE-GN|.LGFGI------SPE
pot-3.1     1                                     ---I-E-GN|.LGFGI------SPE
tom-1.1     1                                     ---T-E-GN|.LGFGI------SP-
tob-1.1     1                                     ---TL----|R---GV-----E-KN
tob-2.1     1                                     ---TKE-GN|.F--GI----Q-TPD

Dom-2; pot-1    NRICTNCCAGYKGCNYYSANGAFICEGESDPKK|PKACPRNCDP|HIAYSKCPRSEGKSLIY

pot-1.2     24  ------------------------------|----------|----------------
pot-2.2     24  ----------------------------Q-----|-----L----|----------------
pot-3.2     24  -P--------------------------Q-----|-----L----|----------------
pot-4.1     1    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~N|-NV------T|N------L-              51
pot-5.1     1    P--------------------------Q-----|-----L----|----------          52
tom-1.2     24  -P--I---S---------NSF-K----------R|-N--TF----|N----R----Q------
tom-2.1     1   D---------T---K-F-DD-T-V-------RN|----------|R---GI--L-          53
tob-1.2     25  D---------T---K-F-DD-T-V-------RN|----TL----|R---GV-----E-KN
tob-1.3     85  D---------T---K-F-DD-T-V-------RN|----------|R---GI--LA-E-KN
tob-1.4     145 D---------K---K-F-DD-T-V--------N|----------|R---GI--L--E-KN
tob-1.5     205 D---------K---K-F-DD-T-V--------N|----------|R---GI--L--E-KN
tob-1.6     265 D---------K---K-F-DD-T-V-------RN|----------|R---GI--L--E-KN
tob-2.2     24  DP---T---------------T-----S----N|-NV--QF---|D-----------ETI-N
tob-2.3     84  PTG--T--T-----Y-FGQD-E-V-------..E|--S-TTE---|RV-TIS--F-GLVKI
egg-1.1     1    Q--------R---S-F-ED-T---K---N-EN|---------G|R---GI--L-          52

Dom-2; pot-1    NRICTNCCAGYKGCNYYSANGAFICEGESDPKK|PKACPRNCDP|HIAYSKCPRSEGKSLIY

pot-1.3     84  PTG--T--T-----Y-FGK--K-V-------..E---NMYPAM                     123
pot-2.3     84  PTG--T--T-----Y-FGK--K-F-------..E---NMYPAM                     123
pot-3.3     84  PTG--T--T-----Y-FGK--K-V-------..E---NMYPAM                     123
tom-1.3     84  PTG--T--T-----Y-FGKD-K-V-------..E---NMYPVM                     123
tob-1.7     325 D---------K---K-F-DD-T-------EYASKVDEYVGEVENDLQKSKVAVS          353
tob-2.4     140 -QE-I---NAD---EL-DND-SL--T-GEPQSAA                              174
```

B

```
                                  ┌──────────┐
NRICTNCCAGYKGCNYYSANGAFICEGESDPKK|PKACPRNCDP|HIAYSKCPRSEGKSLIY
DPG IT  SAD   KLFGKD SLV T Q N RN| NV TLE GN|RLGFGR LLAQEEPE N
PTE     T K   Y  DDF T   K S   ER|  S IKF  T|NF TIS  F GLVKN
 Q      N R   S  NN  K           E|    Q   G|DV   V      STD
          T    E   Q  E           |    T    |     I      T I
               E                  |    F     |
               S                  |          |
                                  └──────────┘
```

Figure 2. A: Amino acid sequence comparison of PI2 proteins that were deduced from genomic clones, cDNA clones and protein sequences isolated from solanaceous plants. Abbreviations: pot-1, Jongsma *et al.* (1995a, this thesis); pot-2, Keil *et al.* (1986), Sanchez-Serrano *et al.* (1986); pot-3, Thornburg *et al.* (1987); pot-4/5 (protein: PTI/PCI-1), Hass *et al.* (1982); tom-1, Graham *et al.* (1985); tom-2 (protein: TTI); tob-1, Atkinson *et al.* (1993); tob-2, Balandin *et al.*, 1995; egg-1, Richardson, 1979. All sequences were compared to the second domain of pot-1. The active domain contains those residues that directly interact with the proteinase molecule. Dots indicate deletions and hyphens indicate identical residues. P_1-P_1' indicates the peptide bond cleaved by the inhibited proteinase molecule. **B:** The variation in amino acid residues at each position of the core domain of PI2. The rectangle contains the proteinase contacting residues

Prospects of phage display

The future importance of phage display as a tool for generating and isolating novel specificities of plant PIs against the plethora of insect pests of plants will depend on the functional display on phages of also other PIs than PI2, on structural information about the proteinase-PI interaction, and on technical improvements of the method. These aspects are addressed below.

The success of attempts to modify proteins and to select desired characteristics by phage display fully depends on the functional display of the wildtype protein. Functional display is not always achieved after fusion to the *N-terminus* of gene 3 protein, however. Such negative results might limit the application of phage display in the area of proteinase inhibitors. In that regard it is important that recently alternatives were published that allow fusion to the *C-terminus* of other proteins. In the first system, a protein is fused to the C-terminus of fos-protein, in a phagemid vector which also expresses jun-protein fused to gene 3 protein (Crameri and Suter, 1993, Crameri *et al.*, 1994). During phage assembly in the periplasm, jun fused to gene 3 protein and fos fused to any other protein bind covalently by disulphide bridge formation. The secreted phage particles subsequently display C-terminal fos-protein fusions on their surface, as if they were normal N-terminal gene 3 protein fusions. In the second system, proteins were directly fused to the C-terminus of a different minor coat protein, encoded by gene 6 (Jespers *et al.*, 1995). Thus, several alternatives exist to achieve the functional display of proteins on phages, to select mutants with improved properties.

The (partial) randomization of the amino acids of the inhibitor molecule involved in binding the proteinase molecule requires a detailed knowledge of which residues are important for the interaction. Conserved amino acid positions may be of structural or biological importance and should be maintained. It will most likely be more efficient to randomize the less conserved positions in the binding region. The information about amino acid residues that are appropriate for mutation can be derived from crystal structures of inhibitor-proteinase complexes, and from protein alignment of all members of a certain PI gene family. Such data are available for many plant and insect proteinase inhibitors and can be used for a randomization strategy. Examples of the strategies followed for the bovine pancreatic trypsin inhibitor, which occurs in vertebrates and insects, have been described. Roberts *et al.* (1992) restricted the randomization mainly to 2-5 naturally occurring amino acid residues at the positions P_1-P_4'of the binding loop (numbering as in Fig. 2 and chapter 1, Fig.1 according to Schechter and Berger, 1967). In this way they were able to isolate inhibitors that were much more active (K_d = 1 pM against neutrophil elastase) than naturally occurring variants, and only needed to construct a library of 1000 different protein sequences. Dennis and Lazarus (1994a,b) made three separate libraries of up to 3.2 million different protein sequences by fully randomizing the positions [P_5,P_3,P_1,P_3',P_4'], [P_5,P_4,P_3,P_1,P_{24}'], or [P_1,P_1',P_2',P_3',P_{19}'], except for the P_1 position of the last two libraries, which was only allowed Lys or Arg. Inhibitors with 10 to 30-fold better dissociation constants than the wildtype protein were isolated. Residues P_5-P_4' were chosen for randomization because they directly contacted the proteinase molecule. The cysteine residue at position P_2 was left unchanged as it is fully conserved and involved in disulphide bridge formation essential for the structure and stability of the protein. The randomization of the P_1 residue was limited to basic residues because this is the known specificity of Factor VIIa. The buried residues P_{19}' and P_{24}', immediately below the proteinase binding loop, indirectly

influenced the structure of the binding loop and by replacing it with other residues the inhibitor affinity for the proteinase was improved 40-fold. Similar considerations will also apply to the randomization of PI2 residues.

The known PI2 genes (Fig.2) possess variable active domains, but with two conserved cysteine residues. The cysteine residues have a structural role in the formation of disulphide bridges as revealed by the crystal structure (Chapter 1: Fig. 1), and would be excluded from randomization. The crystal structure of a PI2-proteinase complex (Greenblatt *et al.*, 1989; see also: Nielsen *et al.*, 1994) provides information about the number of Van der Waals' interactions (affinity) between the two proteins and points out the residues that directly contact the proteinase molecule. Table 1 lists which inhibitor residue contacts which proteinase residues at how many points and *vice versa*. It demonstrates that the P_1-residue, Leu38I in the top row of table 1, is contacted by 10 different proteinase amino acid residues listed in the left column, and is responsible for almost a third of the intermolecular contacts (27 out of 93). This relatively high contribution of the P_1-residue to binding reflects the specificity of most proteinases for cleavage behind specific amino acid residues. However, as indicated in the table a stretch of eight other inhibitor residues contributes 66 additional intermolecular contacts. This loop has been indicated on the proposed crystal structure of PI2 (chapter 1: Fig.1) and is responsible for the inhibitory property of the protein. Amino acid residues that directly contact the proteinase molecule are candidates for randomization. If the affinity achieved by changing these residues would not be satisfactory, changing residues buried directly below the residues that contact the proteinase can improve binding (Dennis and Lazarus, 1994a,b).

After (partial) randomization of a number of amino acids the enrichment factor of functional phage over non-functional phage is critical for the rapid and succesfull isolation of functional phages. With PI2 we achieved a 100-fold enrichment per panning round, but this factor can be improved enormously by the use of non-infectious phage with defective gene 3 protein incapable of infecting *E.coli*. In this system of Dueñas and Borrebaeck (1994) a wt gene 3 protein is coupled to the antigen (proteinase) and added to the non-infectious phage library in low dilution. Addition of *E.coli* leads to selective infection of those inhibitor phages capable of binding the antigen (proteinase) coupled to gene 3 protein. In a single step a 100,000-fold enrichment of functional phages was thus achieved. This enormous selectivity greatly enhances the chances of selecting the proteins with the desired characteristics. The main bottle-neck remaining is the transformation frequency of ligation mixtures into *E.coli*, which limits the size of most libraries to about 10^7-10^8. Recently, an improvement in this regard was marketed by the company Stratagene. They sell a method for introducing larger filamentous phagemid libraries into *E.coli* by using SurfZAP™ lambda vectors.

The phage display method was recently shown to also allow the selection of genes from cDNA libraries displayed on phages (Crameri *et al.*, 1994; Jespers *et al.*, 1995). This created the novel possibility to select PIs not only from synthetic but also from natural variation. A strategy to first look for natural genes with activity for a certain proteinase via phage display of cDNA libraries greatly simplifies and accelerates the classical protein-to-gene approach. Thus, phage display provides, also in a broader context, a method superior to classical methods for isolating a PI gene encoding a protein with specificity for a selected insect proteinase.

Table 1. Summary of intracomplex van der Waals' contacts[1] between Potato Chymotrypsin Inhibitor I (PCI-1) and *Streptomyces griseus* Proteinase B

	Pro33I[3]	Lys34I	Ala35I	Cys36I	Pro37I	Leu38I	Asn39I	Cys40I	Pro42I	Sum
	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_4	
Ser38									2	2
Thr39								1	3	4
Gly40								2	1	3
Arg41							2	10		12
Cys42[4]							1			1
His57[4]					2	1	3			6
Val169			1							1
Tyr171		1	3	4	5					13
Ala190						1				1
Glu191						3				3
Pro192						3	1			4
Gly193[4]						4	1	2		7
Asp194[4]						1				1
Ser195[4]						10	2			12
Ser214					1	1				2
Gly215				2		2				4
Gly216		1	2	6		1				10
Ser217	3	3								6
Gly219	1									1
Sum	4	5	6	12	8	27	10	15	6	93

[1] The numbers in the table are the number of observed intracomplex van der Waals' contacts, which is a measure of the degree of surface complementarity, i.e. affinity, between the two molecules.

[2] Data were taken from Greenblatt *et al.* (1989)

[3] Residues followed by I indicate the inhibitor residues

[4] The underlined amino acid residues are absolutely conserved among all proteinase of the chymotrypsin superfamily

Conclusion

Insects are unable to synthesize a number of amino acids themselves, and are, therefore, fully dependent on the efficient breakdown of plant protein in their digestive tracts. The availability of sufficient amino acids is essential for growth (Bernays and Woodhead, 1984; van Loon, 1988; Burgess *et al.*, 1994; Orr *et al.*, 1994), but also for the production of a large offspring (Deloach and Spates, 1980), and for the sclerotization of cuticular

structures (Anderson, 1985; Mollema and Cole, *in press*). Plant protein is degraded by secreted gut proteinases, but the conserved structure of proteinases has enabled plants to evolve a general defense strategy based on the inactivation of proteinases with inhibitor proteins. This thesis provides evidence that some insects have found a way around this defense by inducing the synthesis of proteinases that are insensitive to plant PIs.

In previous reports of insect resistant transgenic tobacco plants expressing either the cowpea trypsin inhibitor (Hilder *et al.*, 1987) or tomato or potato PI2 (Johnson *et al.*, 1989; McManus *et al.*, 1994) weight reductions of 50% were achieved, which indicate small developmental delays of only 1-2 days. Such small delays are not effective, although moderate effects on life history parameters are amplified over time (Yano *et al.*, 1989). If, for example, population growth of thrips (*Frankliniella occidentalis*) is entered into a mathematical model, which assumes that a certain PI can reduce fertility (3 female eggs/day) and delay development (18 days) by 50%, than the model predicts, that the size of the insect population would be limited to 2% of the control population after 90 days (unpublished results). This level of insect control would be very attractive in many types of agricultural practice, and, in principle, can be achieved with the application of specific PIs. The actual potential of PIs for insect resistance is even greater, however. With better PIs, that reduce gut proteolytic activity more effectively *in vivo*, much stronger negative effects on development, mortality, and fertility of the insects can be expected. This will require a large, dedicated effort specifically aimed at a certain insect-plant combination, however.

In conclusion, the potential of PIs for insect resistance is only beginning to unfold, and must be exploited with the specific aim of complementing the plant's PI defense by introducing PIs into plants that are specific for insect proteinases which are not inhibited by endogenous plant PIs. A unique new development in this regard is the opportunity to specifically engineer insect resistance genes with novel specificities that are selected by phage display. Also in the field of entomology, important research can be done, as it will be of broad ecological, biochemical, and practical interest to understand in which way, and at which rate, proteinase genes of insects have, or can become less sensitive to proteinase inhibitors.

SAMENVATTING

Herbivore insekten zuigen en vreten aan planten en de aarde zou niet groen zijn indien planten zich hiertegen niet met succes zouden verdedigen. Er is een breed scala aan verdedigingsmechanismen bekend, die zich op morfologisch of biochemisch niveau manifesteren. In dit proefschrift werd gekeken naar de wijze waarop veel plantesoorten proberen hun voedingswaarde te verminderen door eiwitverteringsenzymen (proteïnases) in de darm van insekten te remmen. Zaden (bv. granen en bonen) en knollen (bv. aardappels) bevatten vaak hoge concentraties proteïnase remmers (PI's), terwijl PI's bij vraat ook in de bladeren van veel planten geïnduceerd worden (Hoofdstuk 1). Toch kunnen sommige insekten zich ongehinderd op bepaalde planten vermeerderen. De oorzaak van deze schijnbare ongevoeligheid voor PI's werd achterhaald door zowel de PI-inductie in de plant als de proteïnase activiteit in de darm van insekten te analyseren. Het proefschrift verdedigt de stelling dat een succesvolle toepassing van PI's in transgene planten, voor het verkrijgen van resistentie tegen insekten, afhangt van de introduktie van PI's die actief zijn tegen insekteproteïnases, die niet geremd worden door de remmers van de planten zelf.

Hoe verdedigen planten zich tegen vraat door insekten?

Wanneer jonge rupselarven aan tabaks- of tomatebladeren gaan vreten, worden hoge PI-concentraties binnen enkele dagen geïnduceerd (Hoofdstuk 2 en 3). In de volwassen tomate- en tabaksplanten die voor het onderzoek gebruikt werden, vond PI-inductie uitsluitend in de verwonde bladeren plaats. Dit verschilt met eerder onderzoek aan zaailingen, waar PI's ook in de niet-verwonde plantedelen geïnduceerd werden. Een niet-verwond blad bereikt wel een stil alarm, waardoor de PI-inductie twee keer sterker is, wanneer een dergelijk blad ook verwond wordt. Het stille alarm toont aan, dat in volwassen planten het vermogen voor systemische signalering niet wegvalt, maar dat de systemische inductie van PI's in niet-verwonde bladeren niet meer plaatsvindt. Mogelijk zit hier een functionele reden achter. Veel schade aan planten die veroorzaakt wordt door insekten beperkt zich ten slotte aanvankelijk tot slechts enkele bladeren. De plant zou zichzelf dan schade doen, wanneer het een groot deel van de energie en voedingsstoffen, die het ter beschikking heeft voor groei en bloei, zou investeren in het mobiliseren van een afweer in de hele plant zodra ergens een geringe hoeveelheid schade optreedt. Juist de induceerbaarheid van deze genen geeft aan dat planten zuinig in afweer wensen te investeren en dan is het een kwestie van verstandig beheer om een kleine, kwetsbare zaailing volledig te beschermen, maar een grote, volwassen plant alleen daar waar de vreters zich bevinden.

De experimenten toonden aan dat in tabaks- en tomateplanten binnen 24 uur effectieve PI-niveaus geinduceerd worden. Dit suggereert dat het nut van transgene PI's met name gezocht moet worden in een hogere specificiteit voor insekteproteïnases en minder in het feit dat ze constitutief tot expressie gebracht worden.

Hoe wapenen insekten zich tegen de afweer van planten?

De wijze waarop insekten reageren op geïnduceerde of transgene PI's in tabak werd bij rupsen van de floridamot, *Spodoptera exigua*, geanalyseerd (Hoofdstuk 4). Aangetoond

werd dat de PI's de proteïnase activiteit met ca. 30% verlagen, maar dat een verdere vermindering van het vermogen om plante-eiwit af te breken verhinderd wordt door een twee- tot drievoudige stimulatie van de synthese van PI-ongevoelige darmproteïnases. Dankzij de overschakeling op PI-ongevoelige proteïnases kunnen de rupsen zich toch volledig normaal ontwikkelen. Dit mechanisme van adaptatie werd ook aangetoond voor Coloradokeverlarven op aardappelplanten (Bolter en Jongsma, *in druk*) en verklaart mogelijk waarom veel insekten relatief ongevoelig zijn voor PI's.

Tot nog toe werden de effecten van PI's vanuit een ander model verklaard. Broadway en Duffey hadden met dezelfde soort remmer en dezelfde rups al in 1986 waargenomen dat *in vivo* de proteïnase activiteit in de darm van de larven eerder verhoogd dan verlaagd werd, ondanks het feit dat die remmers in staat waren *in vitro* 80% van de activiteit te remmen. Op grond van die waarneming veronderstelden zij dat deze insekten proteïnases zo sterk overproduceerden dat er een overschot van niet-geremde activiteit ontstond. Zij schreven een geringe groeiremming vervolgens toe aan de overproduktie van proteïnases en niet aan de verlaging van de proteïnase activiteit. Deze hypothese leek voor de hand liggend, omdat zij bij zoogdieren de verklaring vormde voor groeiremming veroorzaakt door PI's. Zij lieten echter na (net als de zoogdieronderzoekers overigens!) om de proteïnase activiteit van rupsen, blootgesteld aan hoge PI-concentraties, opnieuw te toetsen op hun gevoeligheid voor PI's. Daardoor zagen zij niet dat die gevoeligheid verdwenen (geremd) was, omdat de geremde proteinases vervangen waren door proteïnases die ongevoelig zijn voor deze PI's.

De resistentie van insekten tegen PI's door induktie van de synthese van PI-ongevoelige darmproteïnases vormt een belangrijk obstakel om deze vorm van resistentie tegen insekten m.b.v. biotechnologie bij planten te introduceren. Het blijkt noodzakelijk te zijn om het PI-afweermechanisme van de plant aan te vullen met PI's die darmproteinases remmen die ongevoelig zijn voor de PI's van de planten zelf. Vooralsnog ontbreken echter de PI's met die speciale eigenschappen. Zulke PI's kunnen misschien geïsoleerd worden uit niet-gerelateerde planten. In dit proefschrift wordt voorgesteld dergelijke PI's niet meer uit planten te isoleren, maar bestaande PI's in het bindende domein willekeurig te muteren en vervolgens te selecteren met een techniek die kortweg faag display wordt genoemd.

Hoe isoleer je proteïnase remmers met gewenste specificiteiten?

De klassieke methode om proteinase remmers te isoleren met een bepaalde gewenste activiteit vereist eerst de zuivering van proteïnase remmers uit plantmateriaal, vervolgens toetsing van de activiteit tegen gezuiverde insekteproteïnases, en tenslotte isolatie van het bijbehorende gen door de aminozuurvolgorde van het eiwit te bepalen om op grond daarvan een DNA sequentie af te leiden. Behalve dat deze aanpak omslachtig is, is het dilemma van een dergelijke benadering de vraag of dergelijke remmers wel natuurlijk voorkomen in planten. Proteïnase remmers kunnen namelijk gegroepeerd worden in een beperkt aantal genfamilies, waarvan het domein dat bindt aan het proteïnase vaak sterk variabel is. Dit suggereert dat deze remmers onderling zullen verschillen in specificiteit voor het scala van verschillende proteïnases aanwezig in de darmen van herbivore insekten. De variatie is echter sterk beperkt door het feit dat elk plantegenoom (de totale genetische informatie aanwezig in de kern) naar schatting slechts 100-200 verschillende proteïnase remmergenen bezit.

In dit proefschrift wordt aangetoond dat het mogelijk is proteinase inhibitor II (PI2) functioneel op het oppervlak van bacteriofagen te exposeren. Wij waren vervolgens in staat om op die wijze een functioneel PI-gen met een factor 323.000 te zuiveren door binding aan

proteïnases van runderen (Hoofdstuk 6). Met behulp van gentechnologie kunnen van een bepaald PI-gen miljoenen licht verschillende PI-genen gemaakt worden en de faag display methode biedt nu de mogelijkheid om daaruit het gewenste, actieve gen te selecteren. In dit proefschrift wordt daarom voorgesteld actieve PI-genen niet uit de natuur te isoleren, maar zelf te maken. Het hele proces om remmers van proteïnases te maken en te selecteren gaat als volgt in zijn werk: Eerst wordt het DNA van een natuurlijk PI-gen in het bindende domein willekeurig gevarieerd. De resulterende kunstmatige verzameling van PI-genen wordt in een circulair stuk DNA (faagmide) gezet, dat getransformeerd wordt naar een bacteriestam (*E.coli*). Co-infectie van de bacterie met een zgn. helperfaag leidt ertoe dat een faagmide verpakt wordt als een faagpartikeltje (een bacterievirus) dat uitgescheiden wordt in het medium. Het bijzondere van deze faagpartikeltjes is vervolgens, dat het PI-eiwit, waarvoor het PI-gen codeert, onderdeel is geworden van het eiwitverpakkingsmateriaal van de faag. In de grabbelton van faagpartikeltjes die gemaakt wordt, is het dus mogelijk al aan de verpakking te zien wat voor gen erin zit! Op biochemisch niveau vertaalt zich dat in de mogelijkheid om PI's (eiwitten) op grond van hun insekteproteïnase-remmende eigenschappen te zuiveren en tegelijkertijd het coderende PI-gen (DNA) in handen te krijgen.

In vergelijking met de klassieke isolatie van PI's biedt de faag display methode veel voordelen: (i) Een eenmaal geconstrueerde bank van 10-100 miljoen licht verschillende PI-genen kan altijd hergebruikt worden voor het isoleren van andere specificiteiten; (ii) de variatie is vele malen groter dan aanwezig in de natuur, waarmee de kans op het isoleren van een gewenste specificiteit aanzienlijk toeneemt; en (iii) de methode biedt een enorme tijdwinst, aangezien de isolatie van een functioneel PI gen bij faag display een kwestie van weken is, terwijl de klassieke benadering al gauw een paar jaar vergt.

Welk perspectief hebben proteïnase-remmers als resistentiefactoren tegen insekten?

De afweer tegen insekten en andere herbivoren, op grond van PI's, is in planten ontwikkeld tot een hoge graad van perfectie. Proteïnase remmers komen alleen tot expressie, daar en wanneer ze nodig zijn. Biotoetsen op artificieel dieet gesupplementeerd met PI's geven aan, dat PI's in sommige gevallen bij hogere concentraties de groei van insekten vrijwel stil leggen en de mortaliteit kunnen verhogen. Het was aantrekkelijk om dergelijke natuurlijke resistentiegenen van planten toe te passen in transgene planten voor het verkrijgen van insektenresistentie. Verscheidene onderzoeksgroepen rapporteerden echter onafhankelijk dat dergelijke transgene planten de larvale ontwikkelingsduur vertragen met slechts 1-2 dagen. In ons onderzoek wordt de larvale ontwikkeling op transgene PI-planten zelfs helemaal niet vertraagd. Dit proefschrift toont aan, dat dit veroorzaakt wordt, doordat de huidige plaaginsekten van planten geadapteerd zijn en de synthese van proteïnases induceren die ongevoelig zijn voor zowel de transgene PI's als de PI's van de plant zelf. Het gebruik van PI's die dergelijke proteïnases wel remmen zal deze zwakke plek in de afweer van planten kunnen herstellen. Wanneer dat lukt vormen PI's weer een aantrekkelijke vorm van resistentie tegen de huidige plaaginsekten. De aminozuren die vrijkomen uit plante-eiwit worden namelijk niet alleen geïnvesteerd in lichaamseigen eiwit tijdens de groei (van belang voor de ontwikkelingsduur) maar ook in de produktie van nageslacht (van belang voor de vruchtbaarheid) en de vorming van een sterk uitwendig skelet (van belang voor de mortaliteit). Indien PI's de ontwikkelingsduur van bv. trips met 50% verlengen en de vruchtbaarheid met 50% verminderen, dan beperkt dit volgens modelberekeningen binnen 90 dagen de schade op transgene gewassen tot 2% van de controleplanten. Om dergelijke

resultaten te bereiken is echter een grote, gerichte inspanning noodzakelijk.

Het potentieel van PI's is veelbelovend, maar sommige plaaginsekten bezitten induceerbare resistentie tegen de PI's van hun waardplant. In het laboratorium zijn we nu in staat het proces van evolutie te imiteren en in principe in korte tijd werkzame PI-genen te isoleren, en staan we voor de uitdaging hiermee de afweer van planten tegen plaaginsekten aan te vullen. Tegelijkertijd is het van ecologisch, biochemisch en praktisch belang meer te weten te komen over de wijze waarop, en de snelheid waarmee insekten resistent worden tegen proteïnase-remmers van planten.

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NAWOORD

Na twee jaar filosoferen over wetenschap was het wennen om in 1989 op het Ital weer achter een labtafel te staan. Het clubje van destijds -Bert Visser, Guy Honee, Theo van der Salm en Ellie Munsterman- vormde echter een hechte eenheid, die mij welwillend opnam in de fantastische sfeer van het lab. De nieuwbouw aan de Droevendaalsesteeg beroofde ons helaas van wat onvervangbaar is gebleken: galmen in de gang (vooral erg voor mij) en de grandeur van bos en oprijlaan.

Het eerste anderhalf jaar onderzoek was niet bedoeld als promotie-onderwerp en leende zich daar ook inderdaad niet voor. Het aanvankelijke optimisme dat proteinase remmers (PIs) een gemakkelijke toepassing in de moleculaire resistentieveredeling van planten zouden kunnen vinden, bleek al gauw gebaseerd op drijfzand. Het was dan ook uitzonderlijk dat een toepassingsgericht instituut als CPRO-DLO destijds de visie had dergelijk fundamenteel onderzoek toch in eigen formatie uit te willen voeren. Willem Stiekema was daar in eerste lijn verantwoordelijk voor. Zijn houding gaf blijk van het inzicht dat onderzoek altijd onvoorspelbare uitkomsten oplevert, die soms interessanter zijn dan het resultaat dat je nastreefde. Dat hij daar vrede mee had, en zelfs enthousiasme voor kon opbrengen, heeft mij moreel gesteund in mijn onderzoek, en verzoend met het natuurwetenschappelijke bedrijf.

Vanaf het derde jaar werd het PI-onderzoek versterkt met Petra Bakker, zonder wier bijdrage dit boekje minimaal een hoofdstuk dunner uitgevallen zou zijn. Petra is in bijna alle facetten van het onderzoek betrokken geweest en dit boekje is daarom ook een beetje van haar. Ik zal haar nog node missen, als ik het straks alleen moet doen. In het vierde jaar voegde ook Jeroen Peters zich bij de club en werd het PI-onderzoek een beetje volwassen. Zijn analyse van de darmproteinases van floridamotlarven is cruciaal voor de voortzetting van het phage display werk en door zijn inspanningen komen de toepassingen van PIs tegen belangrijke plagen als trips snel dichterbij. In diezelfde periode werd Dirk Bosch het nieuwe sectiehoofd. Onder zijn hoede werden de wetenschappelijke kwaliteit en richting van onderzoek en manuscript veel strenger bewaakt dan ik gewend was. Onderzoeksplannen laten zich niet van de ene op de andere dag realiseren en van zijn stap voor stap benadering heb ik veel kunnen leren.

Jan Peter Nap heeft zich verdienstelijk gemaakt met zijn kritische kijk op mijn experimenten, met zijn adviezen op het gebied van de statistiek, en met het aandragen van relevante literatuur. Ruud, Wim, Dion, Fred, Jos, Hilde, Annelies en Bas hebben allemaal zijdelings met het project te maken gehad, al was het maar in de wekelijkse werkbesprekingen. Hun hulpvaardigheid en inzet was onmisbaar.

It is often very hard to obtain the funds to do the research work that one is most interested in, and I was very fortunate, therefore, that Caroline Bolter of Agriculture and Agri-Food Canada (London, Ontario) chose my lab to do some work. The experiments with Colorado potato beetle were quickly arranged with a "minimum" of red tape. As discussed in chapter 7, Caroline's work has provided essential confirmation that insects adapt to proteinase inhibitors. Her witty presence is dearly missed.

Mijn promotor, Ab van Kammen, werd vanaf het derde jaar betrokken bij het onderzoek, en deze betrokkenheid werd substantieel in het laatste onderzoeksjaar en tijdens het schrijven van de verschillende hoofdstukken van dit proefschrift. Wanneer ik dacht de eindversie van een manuscript rond te hebben, wist hij nog feilloos de zwakke plekken aan

te wijzen. Zijn streven naar perfectie is een goede leerschool geweest. Het proefschrift is daardoor duidelijk meer geworden dan de bundeling van een aantal artikelen.

Harry Paul was betrokken in het oorspronkelijke vooronderzoek naar nematoderesistentie, waarvan de resultaten niet in dit proefschrift werden opgenomen. Jan de Jong, Wim Rademaker, Folchert van Dijken en Barbara Gebala werden via het chrysantenproject in het laatste jaar betrokken bij het PI-onderzoek. Chris Mollema wil ik bedanken voor zijn interesse voor het project en voor suggesties om de invloed van PIs ook bij trips en mineervlieg te onderzoeken. De zusterorganisatie IPO-DLO speelde een belangeloze rol door grote hoeveelheden L1-tjes van Spodoptera exigua aan te leveren. Zonder deze Spodoptera kweek van Ronald Jansen en Theo de Vos was het onderzoek stukken moeizamer verlopen. De gewasverzorgers Johan Hulsman, Dick Geurtsen en Hans Jansen op de Goor en Gerrit Stunnenberg en Henk Oosting op de Haaff waren onmisbaar voor de verzorging van de vele planten die ik in de diverse kassen heb gehad. Verder wil ik noemen de bibliotheeksectie bestaande uit Hannie, Hennie, Pieter en Louis, die altijd servicegericht en conscientieus omsprongen met de vele aanvragen voor literatuur of andere informatie. Net zo'n professionaliteit straalde de receptie annex het secretariaat uit.

Tijdens de afgelopen jaren heb ik in samenwerking met anderen verschillende projecten geschreven die hun uitwerking hadden op mijn experimenten. Het plantibody project dat met Jaap Bakker en Arjan Schots geschreven werd, heeft mij geinspireerd om aan phage display te beginnen. Rikus Pomp en Jacolien Zilverentant hebben daarbij een belangrijke ondersteunende rol gespeeld om de zuivering van PI2 mogelijk te maken. Bij Joop van Loon en Huug Schoneveld werd ik me ervan bewust hoe weinig we binnen de afdeling Moleculaire Biologie van insekten afweten: dat wij rupsen voor onze proeven rustig zeven dagen lang in het donker wegzetten wekte duidelijk ontzetting.

Tenslotte het thuisfront: dat ik op gegeven moment vijf dagen werkte met een aanstelling van vier dagen, terwijl ik ook een kind had, zal mij nooit helemaal vergeven worden. Ik zal mij niet verdedigen. Het illustreert de opoffering die in menig opzicht getroost is om dit proefschrift te maken tot wat het is. Corrie, Esther en Pieter zonder jullie was deze periode nooit zo leuk geweest.

Aan mijn ouders draag ik dit proefschrift op. Wanneer je trouwt, twee kinderen krijgt en een promotiebaan hebt, dan voel je je wel eens tekort schieten. De echo van je opvoeding speelt echter je leven lang door je hoofd. Ik hoop, dat jullie in staat zullen zijn ook iets van jezelf in dit proefschrift terug te vinden, want dat zit er wel in.

CURRICULUM VITAE

Maarten Anthonie Jongsma werd geboren op 11 november 1961 te Oldebroek. Hij volgde tot en met het vierde leerjaar gymnasium β aan het Christelijk College Nassau Veluwe te Harderwijk, en ging toen voor 2 jaar naar Canada om op een United World College de middelbare school af te ronden. Aan het Lester B. Pearson College op Vancouver Island behaalde hij in 1980 het Internationaal Baccalaureaat en in datzelfde jaar begon hij de studie Moleculaire Wetenschappen aan de toenmalige Landbouwhogeschool te Wageningen. Juni 1988 behaalde hij met lof het Doctoraalexamen Moleculaire Wetenschappen, biologische orientatie, met de hoofdvakken Erfelijkheidsleer (6 maanden), Moleculaire Biologie (9 maanden) en Wetenschapsfilosofie (ter vervanging van 6 maanden praktijktijd, afgelegd aan de Rijksuniversiteit Utrecht). Juni 1989 werd hij aangesteld als wetenschappelijk onderzoeker bij de Stichting Ital, later het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), alwaar het in dit proefschrift beschreven onderzoek is uitgevoerd als onderdeel van twee projekten. Het eerste, voorbereidende projekt werd gefinancierd door de Programma-commissie Landbouwbiotechnologie (PcLB) en werd uitgevoerd van 1 juni 1989 tot en met 31 december 1990. Dit project had als titel: "Haalbaarheidsonderzoek naar de gevoeligheid van insekten en nematoden voor proteinase inhibitors uit de aardappel" en leverde vooral nieuwe vragen op. Hij werd echter in staat gesteld het projekt tot eind april 1994 als promotie-onderwerp voort te zetten in de vorm van een tijdelijke aanstelling op een CPRO-DLO formatieplaats. In die periode werd het feitelijk hier beschreven onderzoek uitgevoerd. April 1993 werd hij voor 60% betrokken in een extern gefinancierd project om m.b.v. PI's insektenresistentie in chrysant te introduceren. Mei 1994 werd zijn tijdelijke aanstelling omgezet in een vaste benoeming als wetenschappelijk onderzoeker en geeft hij verder inhoud aan de voortzetting van het hier beschreven onderzoek en de begeleiding en uitvoering van het chrysantenproject.