

THE EFFECT OF SELECTED PROTEASE INHIBITORS AND LECTINS IN ARTIFICIAL DIET ON SURVIVAL AND GROWTH OF *ELDANA SACCHARINA* LARVAE

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Abstract

Protease inhibitors, which can inhibit insect digestive enzymes, and carbohydrate binding lectins are potential control mechanisms against herbivorous insects. Larval midgut extracts from *Eldana saccharina* Walker (Lepidoptera: Pyralidae) were assayed for protease activity in the presence of protease inhibitors in order to determine the dominant protease types (serine, aspartic, metallo- or cysteine). Through the use of protease inhibitors, serine proteases having activity similar to mammalian chymotrypsin/elastase were implicated as dominant. Protease inhibitors of this dominant activity were effective only in combination with lectins. Inhibitors of other protease classes were not effective. The selection and genetic engineering of suitable protease inhibitor and lectin genes into sugarcane are discussed.

Introduction

Protease inhibitors, alpha-amylase inhibitors, lectins and lipoxigenases are four classes of proteins in plants considered to function in defense against attacking insects (Gatehouse *et al.*, 1992; Felton *et al.*, 1994). As single gene products, protease inhibitors and lectins are recognised as candidates for the transfer of protection against insect pests through genetic engineering (Johnson *et al.*, 1989; Gatehouse *et al.*, 1993).

Protease inhibitors are generally low molecular weight proteins that form complexes with proteases, thereby inactivating these enzymes. There are four known classes of proteases: serine, cysteine (thiol), aspartic (carboxyl), and metalloproteases (Barrett, 1986). The potential for a protease inhibitor to be active against a specific insect depends on the class(es) of proteases present in the insect's midgut (Christeller *et al.*, 1992; Murdock *et al.*, 1987).

Lectins are broadly defined as carbohydrate-binding proteins other than enzymes or antibodies with the characteristic property of agglutinating blood or other cells (Goldstein and Poretz, 1986). The finding that plant lectins exert significant toxic or growth-inhibitory effects on insects suggests that they play a role in plant defense against insect attack (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995).

The aim of the present study was to determine and compare the effects of mannose binding *Galanthus nivalis* (snowdrop) lectin (GNA), n-acetylglucosamine binding wheat germ

agglutinin (WGA) and selected protease inhibitors on larval survival and growth of the sugarcane borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae).

Methods

Determination of midgut pH and protease extraction

Guts were excised from 100 fifth instar *E. saccharina* larvae and the contents were expelled into an equivalent volume of 0,18 M KCl. The contents were homogenised and the pH was measured. For protease preparation, guts were excised from 20 larvae. These were homogenised in an equivalent volume of 0,18 M KCl, 0,2 M Tris-HCl pH 8,0. Aliquots were dialysed against a buffer of choice to a final concentration equivalent to 2 guts/ml.

Enzyme assays

Buffers were: Kacetate- acetic acid (pH 4,0-5,5); KH_2PO_4 - K_2HPO_4 (pH 6,0-7,0); 0.1 M Tris-HCl (pH 7,5-8,5); KHCO_3 - K_2CO_3 (pH 9,0-10,5); and K_2HPO_4 -KOH (pH 11,0-12,0). General protease activity was determined using sulfanilamide-azoalbumin or sulfanilamide-azocasein as substrates based on the procedure described by Rymerson and Bodnaryk (1995). The pH at which maximum proteolytic activity occurred was determined using the above buffer systems.

Protease inhibitor assays

The proteolytic activities of midgut extracts were assayed in the presence of the following specific protease inhibitors: the serine protease inhibitors, PMSF (Phenyl Methyl Sulphonyl Fluoride), SBBI (Soybean Bowman-Birk Inhibitor), LBI (Lima Bean Inhibitor) and SKI (Soybean Kunitz Inhibitor); a trypsin inhibitor, TLCK (N α -p-Tosyl-L-Lysine Chloromethyl Ketone); the chymotrypsin inhibitor chymostatin; an elastase inhibitor, elastinal; the serine/cysteine protease inhibitors antipain and leupeptin; the cysteine protease inhibitors, E-64 (epoxysuccinyl-leucylamido- (4-guanidino)-butane), cystatin and IAA (iodoacetamide); the metalloprotease inhibitors, EDTA (Ethylene Diamine Tetra Acetic acid) and phosphoramidate; and the aspartic protease inhibitor, pepstatin-A.

The inhibitor concentrations tested were selected according to the effective concentrations recommended by Beynon and Salvesen (1989).

Diet incorporation bioassays

An oligidic synthetic diet (Atkinson, 1978) was used in protease inhibitor and lectin diet incorporation bioassays.

Bioassayed protease inhibitors grouped by class and their concentrations ($\mu\text{g/g}$ of fresh diet) were as follows:

E64 (cysteine)	1 000 $\mu\text{g/g}$
pepstatin-A (aspartic)	1 000 $\mu\text{g/g}$
SKI (serine)	500 and 1 000 $\mu\text{g/g}$.

The lectins GNA and WGA were added at 250, 500 and 1 000 $\mu\text{g/g}$ of fresh diet.

Assays were carried out in 24 celled plates (2 g diet/cell). Each treatment was replicated four times and each replicate contained six cells. One first instar larva was placed into each cell. Survival and biomass were recorded after three weeks at 27°C.

Results*Protease inhibition*

The pH of expelled midgut contents was found to be 9,9. The optimum pH for caseinolytic activity was found to be 10,5. Protease inhibitors were tested at pH 10,5 and at two other pH values. Since the regions of pH optima for plant or mammalian aspartic and cysteine proteases are around 5,5, for metallo-proteases around pH 7,5, and for serine proteases around pH 8, pH values of 5,5 and 8 were chosen.

Inhibition of *in vitro* *E. saccharina* gut caseinase activity by various inhibitors suggests that chymotrypsin and trypsin-like activities are dominant (Table 1). Cysteine, metallo- and aspartic proteases do not appear to be important. Among the three plant derived inhibitors tested (SBBI, SKI and LBI) some variation in inhibition was observed. SKI may be slightly more effective than SBBI.

Table 1. Inhibition of *Eldana saccharina* gut caseinolytic activity.

Inhibitor	Inhibited protease class	Specificity (if not general)	pH 5,5 % inhibition	pH 8 % inhibition	pH 10,5 % inhibition
PMSF	serine	(some cysteine proteases)	60	61	46
IAA	cysteine		12	0	16
EDTA	metallo		18	15	8
phosphoramidate	metallo- endo-proteases		10	2	1
chymostatin	serine	chymotrypsin	40	37	48
elastatinal	serine	elastase	12	11	17
TLCK	serine	trypsin	15	22	30
cystatin	cysteine		11	9	10
E64	cysteine		10	0	27
antipain	serine/cysteine		45	40	63
leupeptin	serine/cysteine		40	39	28
pepstatin-A	aspartic		13	9	7
SBBI	serine	trypsin / chymotrypsin	33	46	60
SKI	serine	trypsin / chymotrypsin	35	70	64
LBI	serine	trypsin / chymotrypsin	9	14	23

NB: Addition of 5 mM cysteine did not stimulate protease activity (a characteristic of cysteine proteases) in controls.

Diet incorporation bioassays

The protease inhibitors SKI, E64 and pepstatin-A had no significant effect on *E. saccharina* survival or growth when incorporated alone into diet. However, SKI in combination with the lectins WGA and GNA significantly decreased the average individual larval mass for survivors at three weeks (Figure 1).

E64 and pepstatin-A had no such effect in combination with lectins (data not shown). In the case of GNA, and particularly the GNA-SKI combination, larval survival was significantly reduced. None of the WGA treatments significantly reduced larval survival.

Discussion

E. saccharina gut protease activity was found to be dominated by chymotrypsin and trypsin-like activities. SKI appeared to be the most effective of the three chymotrypsin/trypsin inhibitors tested.

SKI potentiated GNA and WGA activities in spite of the latter's reported resistance to proteolytic breakdown (Pusztai *et al.*, 1993). *Bacillus thuringiensis* endotoxin (*Bt*) has been shown to have increased toxicity in the presence of serine protease inhibitors (MacIntosh *et al.*, 1990). The level needed to potentiate *Bt* toxicity was several orders of magnitude less than that needed for protease inhibitor toxicity alone.

GNA appears to be more toxic to *E. saccharina* than WGA at least in terms of larval survival (Figure 1). Pusztai *et al.* (1993) expressed concern over the use of transgenic plants expressing WGA due to its toxicity to mammals at concentrations required for effective insect control. GNA has the advantage of having no mammalian toxicity. Indeed, recent experiments have shown that transgenic plants expressing GNA have increased resistance to sucking insects (aphids and leafhoppers) as well as to nematodes (Hilder *et al.*, 1995; Powell *et al.*, 1995). GNA might therefore confer a measure of resistance to pests and pathogen vectors in transgenic sugarcane.

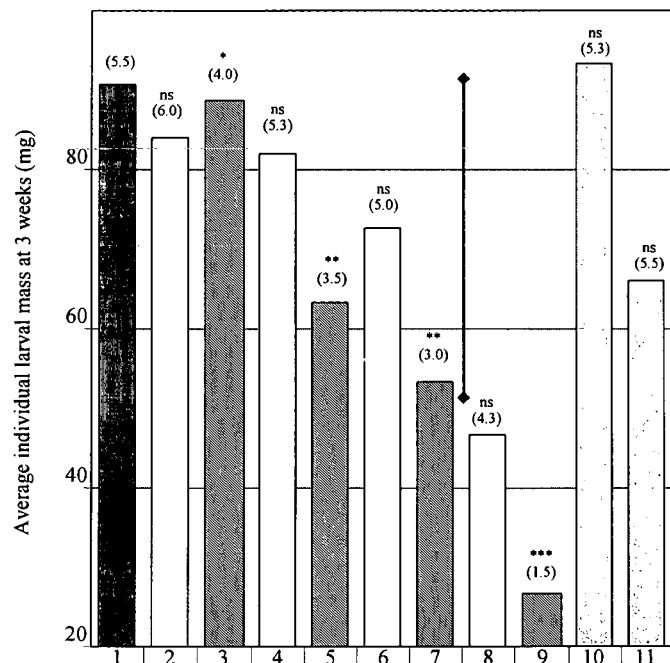


Figure 1. Effect of wheat germ agglutinin (WGA), snowdrop lectin (GNA) and soybean Kunitz inhibitor (SKI) on the survival and growth of *Eldana saccharina* larvae over three weeks (dosage in $\mu\text{g/g}$ of diet).

Bar represents least significant difference for average individual larval mass at $p = 0,05$.

- 1 = Control
 2, 4, 6, 8 = WGA 250 μg , 500 μg , 1000 μg , 500 + 500 μg SKI
 3, 5, 7, 9 = GNA 250 μg , 500 μg , 1000 μg , 500 + 500 μg SKI
 10, 11 = SKI 500 μg , 1000 μg

Average survivors/replicate in parentheses

ns = not significantly different from the control

* = significantly different at $p = 0,05$

** = significantly different at $p = 0,01$

*** = significantly different at $p = 0,001$

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