THE EFFECT OF TRYPSIN INHIBITORS ON GRASS GRUB (*COSTELYTRA ZEALANDICA* (WHITE)) LARVAL GROWTH AND TRYPSIN ACTIVITY

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SUMMARY

The effect of proteinase inhibitors as anti-nutritional factors for grass grub (*Costelytra zealandica* (White)) larvae was investigated. Trypsin, the major grass grub digestive enzyme, was selected as a prime target for inhibition. Trypsin inhibitors with a wide range of *in vitro* enzyme binding effectiveness were incorporated into artificial diet for growth studies. Soybean trypsin inhibitor, which had the highest binding strength, reduced larval growth when added to diet. Elevated trypsin activity was measured in larvae feeding on this diet. Few larvae (one out of 25) reached the second instar when reared on diet containing 0.3% soybean trypsin inhibitor.

INTRODUCTION

A recent survey confirmed that grass grub larvae (*Costelytra zealandica*) are considered by farmers to be the most serious New Zealand pasture pest (Dymock and Ford 1989). Damage from larvae feeding on roots is most serious during autumn and winter. White clover (*Trifolium repens*), the major New Zealand pasture legume, is particularly susceptible. Recent advances in plant molecular biology have made it possible to introduce cloned genes into white clover using the vector *Agrobacterium tumefaciens* (White and Greenwood 1987). The search for single gene resistance factors against grass grub for eventual introduction into white clover began with an investigation of larval gut enzymes by Christeller and Shaw (1989) who identified trypsin as the major gut enzyme and therefore a prime target for inhibition studies. The binding effectiveness of trypsin inhibitors to grass grub gut enzymes was also determined. This is measured as the constant (kd) of the reaction [enzyme] + [inhibitor] $\leftrightharpoons$ [enzyme inhibitor] (Christeller and Shaw 1989). The present study compares the binding effectiveness found *in vitro* with growth of first and third instar larvae on artificial diets containing the same inhibitors. Trypsin activity as a result of feeding on such diets was also measured.

METHODS AND MATERIALS

Trypsin inhibitors (TI) which varied in their *in vitro* effectiveness were selected for incorporation into an agar-based artificial diet containing carrot, Egmont loam, a balance of vitamins, antibiotics, sugars and a base level of 1.5% casein (P. Wigley pers. comm.). The inhibitors, in solution, were added to the agar diet as it cooled to 70°C. Since proteinase inhibitors may themselves act as nutritional proteins, the base diet had bovine serum albumin (BSA) added so that all diets contained equivalent protein levels.

Two experiments measuring the growth of grass grub larvae on the diets are reported here. The first measured the growth of third instar larvae in autumn 1988 and this was followed by an experiment in spring using first instar larvae. In the first experiment trypsin inhibitors were added to the diet so that the final concentrations were 0.02, 0.4 and 1% fresh weight. Weight gains of late second instar grass grub larvae collected from Manawatu hill country in February 1988 and kept at 18°C were measured over 7 weeks by which time they had reached the third instar. There were 25 larvae in each treatment and each received 0.25 g of diet every 5 to 6 days. Larval weight gains were analysed using initial weight as a covariate and were pooled when no concentration effect on larval growth was recorded. Least square means (Lsmeans) for the weight gains were used to test for significant differences between treatments. Chicken egg white TI was not available at the highest concentration so was not included in the analysis. At the end of the experiment the free trypsin (trypsin activity) in the guts of larvae from each treatment was measured by light absorption as optical density/g grub weight (Christeller *et al* 1989).

Larvae used in the second experiment were the progeny of adults which emerged from prepupae and pupae collected from Manawatu sand country in September 1988. Adults were placed in bins containing pre-sieved dampened sand. Eggs were collected and placed in moist vermiculite. Neonate larvae were placed in peat soil for 24 hours before being transferred to individual ice-cube compartments containing 0.25 g of the same agar-based diet and kept at 18°C as in the previous experiment. Trypsin inhibitors were added at a concentration of 0.3% fresh weight of diet. As third instar larval growth on diet containing BSA in the first experiment was not significantly different from growth on some of the test inhibitors, casein was also tested as a supplementary protein at the same level as the trypsin inhibitors. Diet was replenished every 7 days. There were 25 larvae per trypsin/protein treatment which were weighed weekly. The experiment was terminated after 9 weeks. There was no significant effect of initial weight on weight gain so initial weight was not used as a covariate in the analysis. At the end of the experiment the guts were removed from surviving insects and the free-trypsin content measured by light absorption as above.

RESULTS AND DISCUSSION

Growth of early third instar larvae was significantly less on diet containing soybean trypsin inhibitor (SBTI) than on the base diet plus BSA. SBTI and wingbean TI also had the tightest binding efficiencies of the trypsin inhibitors previously measured. Trypsin activity was also elevated in larvae feeding on SBTI (Table 1). Broadway and Duffey (1986) who studied the effect of SBTI on growth of the tomato fruitworm (*Heliothis zea*) and the beet armyworm (*Spodoptera exigua*) also found that it reduced larval growth and elevated trypsin activity. It is not known whether the poor growth recorded is due to inhibition of the digestive enzymes and hence starvation, or the physiological cost of increased enzyme production.

TABLE 1: Weight gain and trypsin activity by third instar grass grub larvae feeding on artificial diet containing trypsin inhibitors (all concentrations pooled within treatments) with a range of binding strengths to grass grub trypsin.

Treatment	Binding constant† Kd (nM)	LS Mean weight gain (mg)	Mean trypsin activity optical density/g/min
Chicken egg white TI	16	89.0*	0.55 a
Limabean TI	0.61	74.4 a	0.67 a
Turkey egg white TI	0.88	73.2 ab	0.74 a
Base + BSA (control)	—	72.7 ab	0.41 a
Wingbean TI	0.087	56.7 bc	0.49 a
Soybean TI	0.12	54.5 c	1.58 b

* Not included in the analysis due to insufficient data

† Binding constants from Christeller and Shaw (1989) — the lower the value, the tighter the binding.

a,b,c: values without a letter in common differ significantly ($P<0.05$).

In the second experiment SBTI was again the most effective first instar growth inhibitor and only one larva reached the second instar after 9 weeks on this diet (Table 2). SBTI, chicken egg white and limabean trypsin inhibitors incorporated into diet resulted in higher trypsin activity in larvae (Table 2).

TABLE 2: Weight gain, survival and trypsin activity of first instar grass grub larvae on diet containing trypsin inhibitors after 9 weeks (25 larvae per treatment).

Treatment	Mean weight gain (mg)	No. survivors	No. reaching 2nd instar	Mean trypsin activity optical density/g/min
Base diet (control)	9.15 ab	20	9	0.64 a
Base + BSA (control)	10.91 abc	17	6	0.53 a
Base + casein (control)	11.72 abc	17	10	0.63 a
Limabean TI	16.54 c	20	15	1.26 b
Chicken egg white TI	9.74 b	18	6	1.55 bc
Soybean TI	3.78 d	19	1	2.64 c

a,b,c,d: values without a letter in common differ significantly ($P<0.05$).

Kain and Atkinson (1977) found that larval density was more sensitive to changes in larval growth rate in summer, when they are young, than in autumn and that live weight is critical for survival only below a certain weight and/or developmental stage. However, it is still not known what part clover roots play in the food requirements of first instar larvae. Wightman (1972) was able to rear first and second instar larvae on root-free diet but growth rate was not as high as that in the field. Also anti-nutritional factors may simply result in extended generation times, rather than a reduction in plant damage. Grass grub larvae have never been reared successfully for a full life cycle on the diet used here and so inadequacies of the diet may have accentuated the effects of protein deficiency caused by ingestion of trypsin inhibitors. Broadway and Duffey (1988) found that, for the beet armyworm, soybean trypsin inhibitor was less toxic when in a diet containing a more nutritious protein.

The potential of proteinase inhibitors as single-gene resistance factors lies with experiments on whole plants. The possibility of proteinase inhibitors being feeding deterrents to grass grub also requires study. Although the proteinase inhibitors tested here are digested by sheep rumen fluid (B. Shaw unpublished data) research on root promoters is continuing to ensure that inhibitors will be expressed only in the roots and hence reduce the risk of adverse effects on stock.

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