DIGESTIVE PROTEASES OF THE GREEN MIRID, *CREONTIADES DILUTUS*

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INTRODUCTION:

The green mirid, *Creontiades dilutus* (Hemiptera: Miridae), is a serious pest of cotton in Australia (Adams and Pyke, 1982). Mirids are sucking insects which feed preferentially on the actively growing points of young plants. The damage they inflict to the tips of pre-squaring cotton and to early squares can result in delayed maturity of the plants and a reduced yield (Bishop, 1980).

Current control of the green mirid relies on early season chemical sprays. The use of chemical insecticides is disruptive to beneficial insect populations as well as being expensive and environmentally harmful. One new strategy for insect control is the use of genetic engineering to produce plants resistant to insect attack. Chemical control of *Heliothis armigera* has already been augmented with the use of cotton plants expressing *Bacillus thuringiensis* toxins (Ali and Young, 1993). A similar strategy for control of the green mirid would be a desirable alternative to chemical insecticides.

One of the difficulties faced when attempting to develop transgenic plants resistant to the green mirid is the lack of information available on the mirid digestive physiology. This lack of knowledge makes it difficult to predict which gene products may be valuable for the development of an efficient transgenic control strategy. Most of our understanding of mirid physiology has come from research on *Lygus* bugs (Hemiptera: Miridae), which are serious agricultural pests in the United States. This research has shown that *Lygus* bugs feed by penetrating the plant tissue with their mouthparts and secreting a watery saliva (Miles, 1972). The saliva contains several

enzymes, specifically proteases, amylases and pectinases, thought to play a role in both penetration of the plant cell and in external digestion of the plant material (Laurema *et al*, 1984). The partially liquefied food is then sucked up through the mouthparts and passed into the gut, where digestion is completed and absorption occurs.

While it is assumed that the green mirid feeds by the same general mechanism as *Lygus*, many details of the green mirid digestive system remain unknown. The broad aim of this work is to gain a better understanding of the digestive system of the green mirid, with the intention of working towards developing transgenic cotton resistant to the green mirid. More specifically, the aims of this work are:

A) To optimise a method to measure protease levels in digestive system of the green mirid.

B) To use this method to investigate the levels of proteases in the green mirid.

C) To characterise the proteases present in the digestive system of the green mirid.

Measuring the levels of protease activity in the mirid digestive system is important for two reasons. Firstly, for an insecticidal gene product to maintain effective control of the pest insect, it must reach its target site within the insect in an active form. This may not be possible if there are high levels of active proteases in the insects gut able to digest the transgenic product. Secondly, the presence of enzymes in the insect digestive system may offer potential targets for enzyme inhibitors used as control agents in transgenic cotton.

RESULTS/DISCUSSION:

A/B) Measuring protease levels;

A general protease assay, using azocasein as the substrate, was used to measure protease levels in the saliva, salivary glands and midgut of the green mirid (Fig 1). This method was modified from Beynon (1989) by adjusting enzyme concentrations

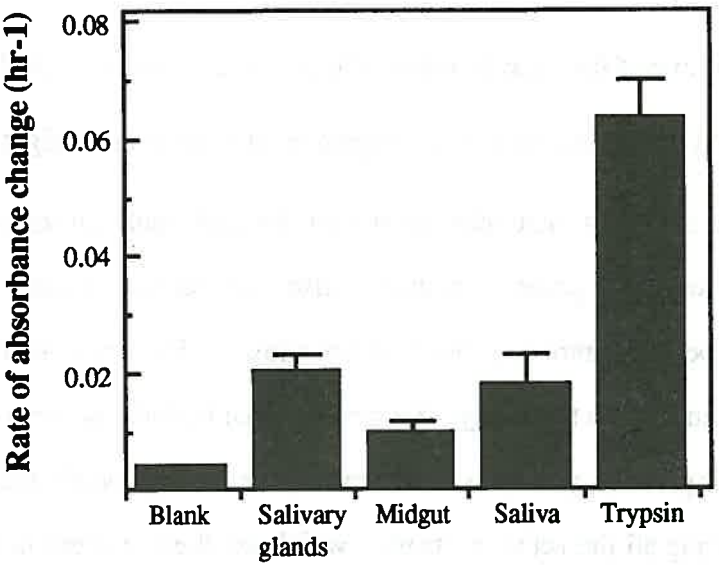

Fig 1: Protease levels (calculated as the change of absorbance at 340nm per hour) present in a blank of buffer only, salivary glands and midgut of three adult mirids, saliva collected overnight from 30 adult mirids, and 1µg of trypsin.

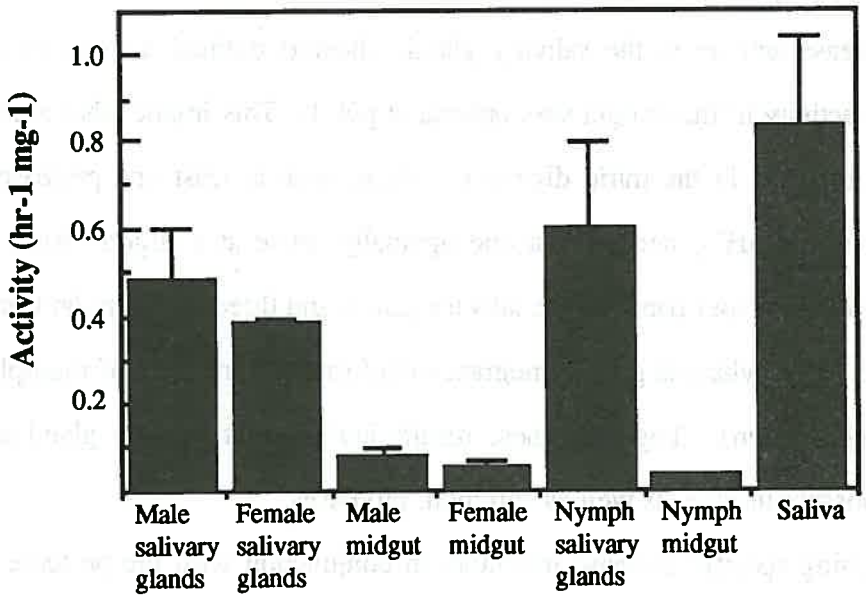

Fig 2: Levels of protease activity (calculated as change of absorbance at 340nm per hour per milligram of protein) compared for adult male and female mirid salivary glands and midgut, fifth instar nymph salivary glands and midgut, and secreted saliva.

and the length of time of the assay to allow detection of the very low levels of proteases active in the green mirid (less than 0.1µg trypsin equivalent in the midgut) (Fig 2).

These results show that fifth instar nymphs and adult mirids of both sexes produce similar levels of protease activity. Also, the salivary glands contain more protease activity per milligram of tissue than the midgut. The origin of the protease(s) cannot be determined from this assay. It is possible that both the salivary gland and gut cells are producing active proteases. However it is also possible that the salivary glands are producing all the active proteases, which are then secreted in the saliva and sucked back into the gut with the partially digested food.

C) Characterisation of the proteases;

Using another general protease assay with haemoglobin as the substrate, the pH optima for the protease(s) present in the mirid digestive system has been examined (Fig 3). Protease activity in the salivary glands showed optimal activity at pH 8, and protease activity in the midgut was optimal at pH 3. This implies that more than one protease is active in the mirid digestive system, with at least one protease optimally active at acidic pH's, and at least one optimally active at a slightly basic pH. The presence of up to four bands in the salivary glands and three in the midgut on protease-detecting polyacrylamide gels (zymograms) confirms the presence of multiple activities (results not shown). Together, these results indicate that salivary gland and midgut tissues possess unique, as well as common, proteases.

Using specific protease inhibitors in conjunction with the protease assay has demonstrated the presence of both serine-like and cysteine-like protease activities in the saliva, salivary glands and midgut of the green mirid (Table 1).

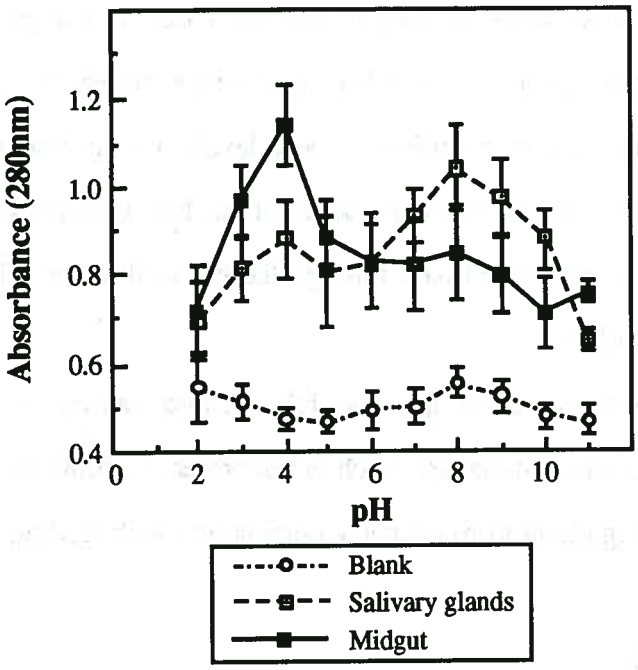

Fig 3: pH optima of mirid proteases. Salivary glands and midgut of ten adult mirids were incubated overnight with haemoglobin in a buffer of known pH. Absorbance at 280nm was then read as an indicator of protease activity.

Sample → / ↓ Inhibitors	Saliva	Salivary glands	Midgut
AEBSF (serine protease inhibitor)	99%	46.7%	13%
Antipain (serine protease inhibitor)		37.2%	15.6
E-64 (cysteine protease inhibitor)	52%	21.1%	21.1%

Table 1: Percentage reduction in protease activity in mirid tissues incubated with specific protease inhibitors.

CONCLUSION:

The secreted saliva, salivary glands and midgut of the green mirid possess low levels of protease activity from at least two active proteases, based on pH optima, zymography, and inhibitor studies. These levels are so low that it is possible an ingested toxin may not be degraded before it reaches its target site. In fact, we have seen that proteins can indeed pass through the gut wall of the mirid and retain activity (our unpubl. results).

The proteases present appear to fall into two classes, serine and cysteine-like. We can now design a rational approach to test protease inhibitors for detrimental effects on the mirid using the in-vitro assays in conjunction with feeding and growth assays.

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