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Binding affinity and larvicidal activity of a novel vegetative insecticidal protein Vip3V

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Abstract. *Bacillus thuringiensis* (*Bt*) is a well-known entomo-pathogen. Strains of *Bt.* that are toxic to a variety of insects belonging to various orders such as Lepidoptera, Diptera and Coleoptra have been identified, their larvicidal proteins isolated, characterized and used extensively in agriculture for protecting the economically important crops against these pests. Recently a novel group of vegetative insecticidal genes, *vip3S* and *vip3V* coding for 789-amino acid (88.5 kDa) protein in *Bacillus thuringiensis* was cloned and expressed in *Escherichia coli* and found to be highly sensitive to most of the agricultural pests and even towards the resistant insects. The binding affinity of these toxins to the receptors of these insect pests and relation between the affinity and larvicidal activity were carried out. Labeling of the toxin proteins with radioactive ^{125}I and preparation of the Brush border membrane vesicles of the receptors were carried out. Analysis of saturation kinetics (K_d) and binding constants (B_{max}) by competition assays revealed direct correlation between the binding affinity of the toxin to the receptor and insect mortality.

INTRODUCTION

Bacillus thuringiensis (*Bt.*), a ubiquitous gram-positive, spore-forming bacterium and mostly forms a parasporal crystal (δ-endotoxins) during the stationary phase of its growth cycle though vegetative phase expression was also reported (Sekar, 1988). This protein (Cry toxin) has larvicidal activity which led to the development of varieties of biopesticides based on *B. thuringiensis* for the control of certain Lepidoptera, Diptera, and Coleoptera insect species (Beegle & Yamamoto, 1992). Thus as an alternative to synthetic chemicals, *Bt.* based pesticides were useful in commercial agriculture, forest management as well as in mosquito control. However, as in the case of chemical pesticides, prolonged use of these proteins has led to the development of resistance problems. *Agrotis ipsilon* (Black cut worm, BCW) is one such agronomically important insect quite resistant to δ-endotoxins. The newly discovered *vip3A* (Estruch *et al.*, 1996), *vipS* (Selvapandian *et al.*, 2001) *and vip3V* (Doss *et al.*, 2002) genes encode about 88.5 kDa proteins that exhibit toxicity towards a wide variety of lepidopteran insect pests, including *A. ipsilon*, *Spodoptera frugiperda*, *Spodoptera exigua*, and *Helicoverpa zea*. MacIntosh *et al.* (1990) have reported that the δ-endotoxins Cry1A(b) and Cry1A(c) possess insecticidal properties against BCW with LC_{50} > 80 µg and 18 µg of diet per ml respectively whereas Vip3 proteins are reported (Estruch *et al.*, 1996) to provide 100% mortality when added at 62 ng of diet per ml. Thus it is 290 fold more lethal in bringing out 100% mortality in BCW than the concentration of Cry1A(c) that is required to cause just 50% lethality (Estruch *et al.*, 1996). Thus the importance of this protein as a very effective second generation pesticide against the common pests had gained attention. In order to understand the biochemical mechanism of the Vip3 toxicity to these pests, the relationship between the

affinity kinetics of the binding to the insect midgut receptor and the mortality was examined.

MATERIALS AND METHODS

Expression of Vip3V

The *B.t.* gene coding for the 88.5 kDa protein was cloned in *Escherichia coli*, expressed, purified (Doss *et al.*, 2002) and labelled with the radioactive iodine (^{125}I). Iodination of the toxin was done based on the iodogen method of Fraker & Speck (1978). Similarly trypsinized and untrypsinized Vip3V, Cry1Ac were also labeled for comparing the binding affinities of each of these proteins.

Receptor Binding and Kinetics

Insect brush border membrane vesicles (BBMV) were prepared (Wolfersberger *et al.*, 1987) from the dissected midguts of the pests (III instar larvae) and allowed to bind with labeled toxin with increasing amount of competitor (Unlabelled protein). Correlation of its specificity and affinity of the specific midgut receptors to the toxin and the susceptibility of these insects were checked.

K_d and B_{max}

Kinetic analysis Cry toxin-receptor binding (Van Rie, 1989) such as equilibrium dissociation constant (k_d) and the maximum binding site concentrations (B_{max}) of vesicle protein were calculated by Scatchard plot using the software GraphPad PRISM.

Bioassay

The *in vivo* pathological, cytological and biochemical changes of susceptible and non susceptible (control) insects (Late neonatal or I instar larvae) and finally the mortality in days that occurred after feeding with the toxin were recorded and photographed.

RESULTS

Pathophysiology

Histological analysis of the susceptible insect midgut section showed shedding of the epithelial cells and finally lysis of the midgut (Fig. 1).

Iodination profile

In the iodination procedure fraction number 12 of the Vip3V had the maximum labeled radioactivity with a specific activity of about 4 mCi / mg (Fig. 2).

Buffer optimization

Among the buffers tested, sodium carbonate buffer, pH 10.0 showed maximum specific binding of Vip3V to the BBMV protein (Fig. 3).

Saturation Binding

Incubation of a fixed amount of labeled toxin with varying amounts of vesicle proteins of tobacco caterpillar (*Spodoptera litura*), diamond back moth (*Plutella xyllostella*) and gram pod borer (*Helicoverpa armigera*)

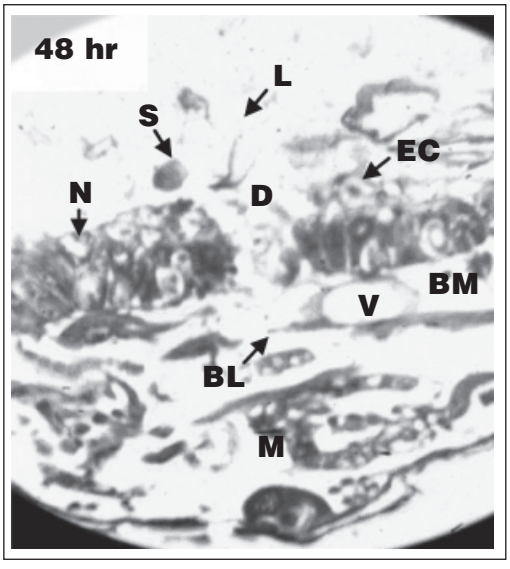

Figure 1. Photomicrograph (4500 X original magnification). Iinsect tissue fixed at 48th hour post-injestion of the Vip3V. Magnified image correspond the following organells;

BM – Separation of epithelial cells from basal membrane. **D** – Midgut – in advanced stage of Disruption, **EC** – Epithelial cells balloon into the lumen, **M** – Loss of definition and Paralysis of the Basal muscle, **S** – Sloughing of epithelial cells, **V** – Basal vacuoles – merging to form extensive vacuolation, **BL** – Basal Lamina, **N** – Apical displacement of nuclei. **L** – Lumen.

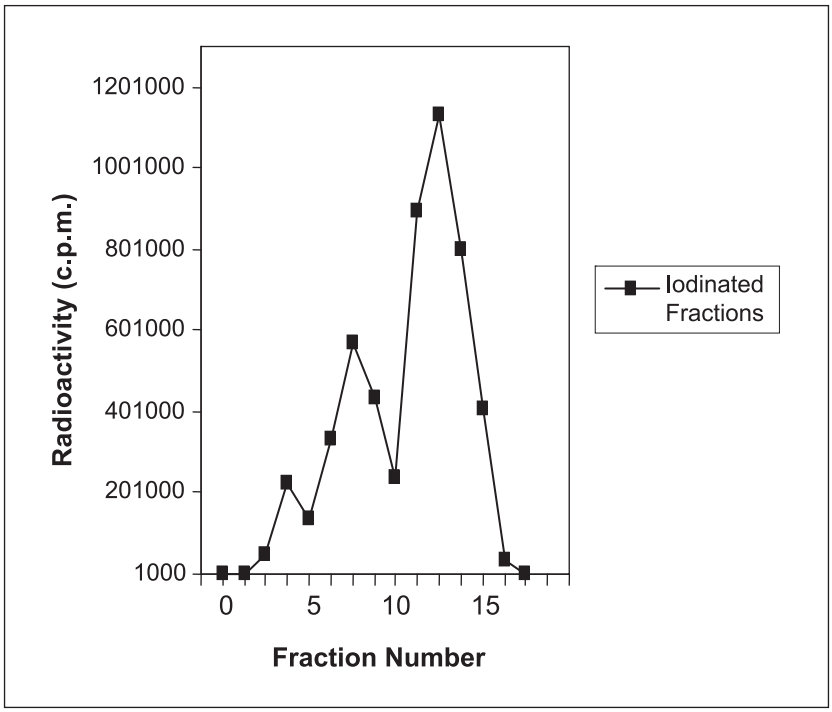

Figure 2. Iodination Profile of the Vip3V protein.
Trypsinized Vip3V labeled with ^{125}I was fractionated on the Sephadex G-50 column and the collected fractions were counted in the gamma counter. The fraction showing the maximum radioactivity (Fraction 12) and specific-binding with the insect BBMV was used for further assays.

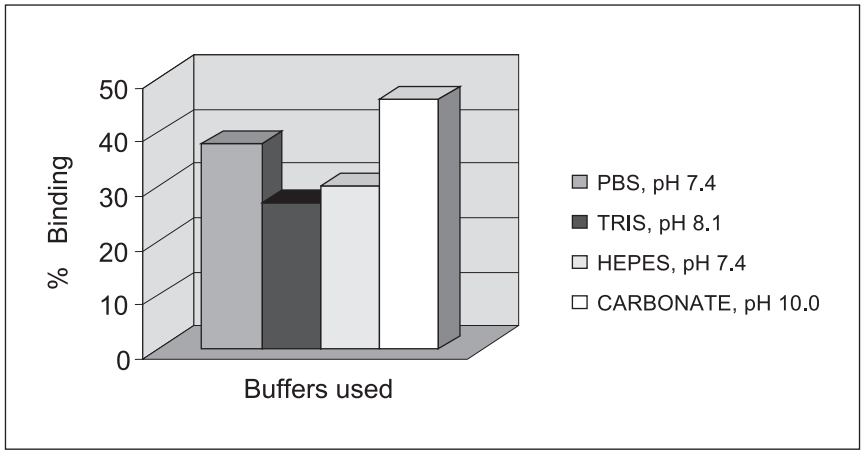

Figure 3. Buffer Selection. Binding assay done with various buffers keeping the other components of the reaction constant. 6 µl (50 µg) of BBMV and 5 µl of the labeled Vip3V toxin ($2 * 10^5$ c.p.m. approx.) were used. The final volume was made upto 100 ìl and incubated for 1 hour at 37°C. Radioactivity in each buffer mix was measured using gamma counter.

showed saturable and specific binding with the Vip3V (Fig. 4). Among BBMV(60 µg/ml) of the insects that showed specific binding, BBMV of tobacco caterpillar (*S. litura*), and Diamond back moth (*P. xyllostella*) showed 48% and 55% binding with the trypsinized and radio-labelled toxin. Gram pod borer (*H. armigera*) showed 30% with the labeled Vip3V (in the absence of the competitor). However, silk worm *(Bombyx mori*) vesicle showed an extremely low specific binding when compared with the basal non-specific binding.

Competition Assays

Competition experiment with fixed vesicular protein and ^{125} I-Vip3V and varying amounts of unlabelled toxin showed that the affinity was found to be more with the trypsinized Vip3V followed by trypsinised Cry1Ac and found to decrese with the un-trypsinized Vip3V, and untrypsinized Cry1Ac in the decreasing order respectively (Fig. 5).

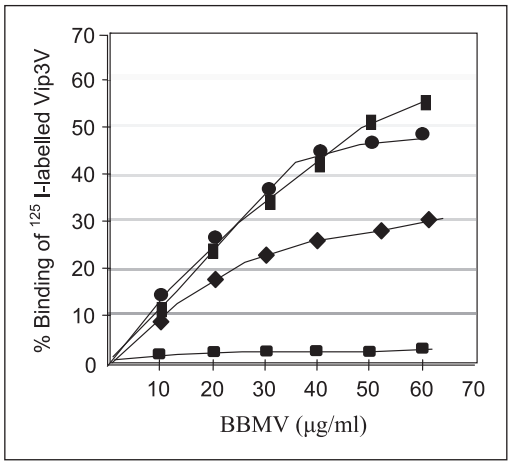

Figure 4. Binding of ^{125}I-labeled Vip3V as a function of BBMV concentrations of;

- TCP, tobacco caterpillar (*Spodoptera litura*);
- DBM, diamond back moth (*Plutella xyllostella*);
- GPB, gram pod borer (*Helicoverpa armigera*);
- BM, silk worm *(Bombyx mori*).

Non-Specific Binding was seen with a 100 – fold excess of unlabeled crystal protein. Each point represents the mean of two experiments.

DISCUSSION

It is well known that the susceptibility or resistance of an insect differs in the steps that are involved in solubilization of the toxin, pH of the gut regions, activation of protoxin to toxin, degree of binding to the receptors (Gill *et al*., 1992) and pore or channel formation (Hofmann *et al*., 1988, Van Rie *et al*., 1989, Lee *et al*., 1992, 1996; Knowles *et al*., 1994).

The pathological, cytological and biochemical changes that occur due to the toxicity of δ-endotoxin on the lepidopteran insects are well known (Aronson & Shai, 2001) and the Vip3V protein is found to produce same damaging effects and symptoms such as cessation of feeding, regrugitation and gut paralysis as seen in the case of Vip3A (Lee *et al*., 2003).

Early works (Hoffman *et al*., 1988; Van Rie *et al*., 1989) employed competition binding studies to demonstrate the correlation between toxin affinity and insecticidal activity. Receptor binding, insect specificity (Van Rie *et al*., 1989), and the correlation between the specificity and

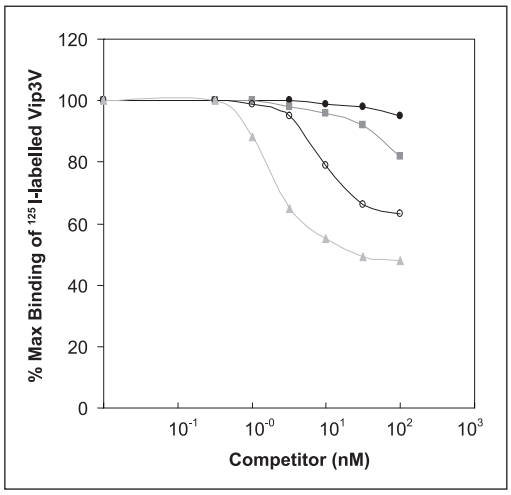

Figure 5. Competitive binding assay using ^{125}I-labeled Vip3V toxin and insect brush border membrane (*Spodoptera litura*) vesicles.
Vesicles (50 µg) were incubated with 0.5 nM of labeled toxin and with increasing concentrations of unlabeled competitor. Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin. Each point represents the mean of two. The legends used are:

- = Trypsinized Vip3V Toxin
- = Trypsinized Cry 1Ac Toxin
- = Untrypsinized Vip3V Toxin
- = Untrypsinized Cry 1Ac Toxin

binding domains for Cry1Aa against *B. mori* were demonstrated (Lee *et al.*, 1992, 2003).

In this study various buffers were tested for maximum toxin binding (with the insect BBMV) and the sodium carbonate buffer (pH 10.0) was found to favor maximum binding of the toxin followed by phosphate buffer (pH 7.4). HEPES buffer (pH 7.4) and Tris (pH 8.1) were found to favor lesser binding of the toxin (Fig. 3).

When the excess of labelled ^{125}I -Vip3V toxin was incubated with varying amounts of vesicular proteins (in BBMV) of *S. litura*, *P. xyllostella*, *H. armigera*, *B. morri*, the three vesicular proteins except the *B. morri* (which is not a susceptible insect), showed parabolic saturable binding. Polyethylene Glycol (PEG) at a final concentration of 10% was found to precipitate the labelled toxin. Thus the radioactivity found in the vesicle – bound toxin and the free toxin in the supernatant were checked and found to correlate with the total radioactivity used.

The % saturation of binding was 48%, 55% and 30% (Fig. 4) respectively for the *S. litura*, *P. xyllostella* and *H. armigera*, though the LD_{50} values for these insects were 45.41, 220 and 325 ng/cm^2 respectively (Table 1). The low specific binding found with the *B. mori* vesicles in saturation experiments is suggestive of low affinity for the protein which may explain the non-susceptibility of this insect to the Vip3V protein (Hofmann *et al.*, 1988).

Competition experiments with labeled Vip3V showed that the binding of trypsinized Vip3V (0.5 nM) protein was saturable in the presence 1-100 nM competitor (inhibition slope) and trypsinzed Cry1Ac with saturable binding up to 0.1 to 1 nM of the competitor (Fig. 5).

The equilibrium dissociation constant (K_d) and the binding site concentration (B_{max}) were calculated using the Scatchard Plot (Fig. 6). The BBMV protein of *S. litura* and *Pl. xylostella* showed the K_d values 0.62 and 1.6 nM and the B_{max} values of 7.2 and 8.2 pmol/mg consistant with their LD_{50} values (45 and 220 ng/cm^2). The lesser the *H. armigera* susceptible insect (LD_{50} value 325 ng/cm^2) showed the K_d value of 2.0 nM and the B_{max} value of 10.8 pmol/mg protein (Table 1).

While previous reports (using Cry1Aa, Cry1Ab and Cry1Ac) had shown that there is a direct correlation between the toxicity and the binding affinity of a few target insect midguts (Hofmann *et al.*, 1988, Van Rie *et al.*, 1989; Lee *et al.*, 1992) inverse quantitative relationship between insecticidal activity and receptor binding have also been found in some insect varieties. Cry1Ab was found

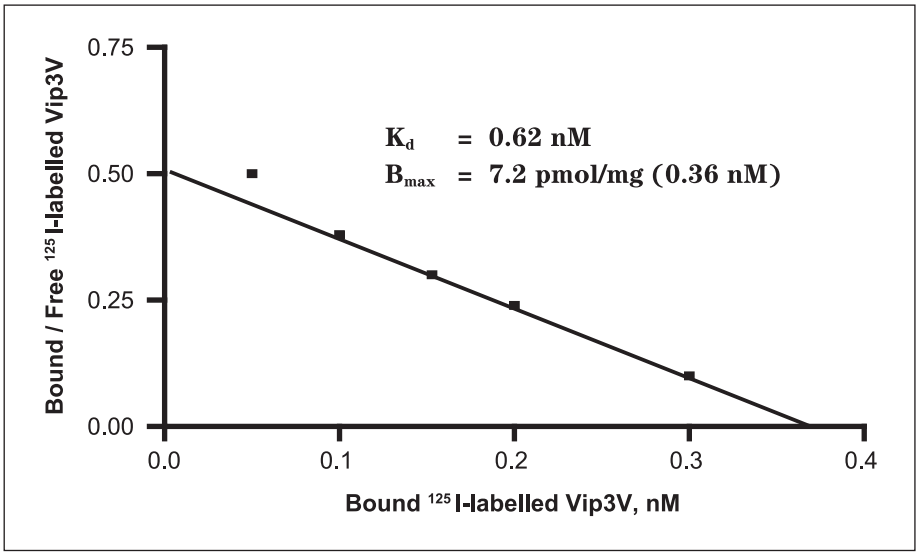

Figure 6. Data from competition between labeled toxin and the unlabeled toxin plotted in Scatchard Plot.

Table 1. Binding of Vip3V to the Brush Border membrane Vesicles of the susceptible insects and LD_{50} values. [a]Late neonatal or I instar larvae. [b,c]III instar larvae. K_d (nM) and B_{max} (pmol/mg) were determined using the software "GraphPad PRISM". Each value is the mean of three experiments performed on two independently prepared batches of vesicles

Sl. No.	Insect BBMV used	LD_{50}[a] (ng/cm^2) 95% Fiducial limits	K_d [b] (nM)	B_{max}[c] (pmol/mg)
1.	*Spodoptera litura*	45.41 (37.4–55.2)	0.62 ± 0.22	7.20 ± 2.20
2.	*Plutella xylostella*	220.73 (99–494)	1.60 ± 0.20	8.20 ± 2.40
3.	*Helicoverpa armigera*	325.20 (119–881)	2.00 ± 0.40	10.80 ± 5.0

more effective against gypsy moth larvae than Cry1Ac against gypsy moth larvae, despite exhibiting a relatively weaker binding affinity than the later.

In the present study the binding affinity and the toxicity of the protein to the susceptible insects were compared. Recently Selvapandiyan, *et al.* (2001) reported that the insecticidal activity of Vip3S resides in the residues 40 to 637. Competition assays in this study showed that typsinized Vip3V and Cry1Ac bound more specifically than the untrypsinized whole toxin molecule. When compared with *S. litura* the most sensitive insect, LD_{50} value of *P. xylostella* increased 4.8 times and there was 2.5 fold decrease in the kinects of binding. The same was true in the case of *H. armigera* (1.4 times increase in LD_{50} value with 1.25 fold decrease) when compared with *P. xylostella*. Thus there seems to be a direct correlation between binding affinities, kinetics and the morbidity.

Though the general correlation need not always hold true and the mechanism of susceptibility in each insect variety may differ from each other, we have shown the usefulness of saturation binding and kinetic studies as a means of a tool for interpreting the effectiveness of the toxin in insect control programmes which as an extension can be used in insect resistance management programmes.

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