Specificity of *Bacillus thuringiensis* δ-endotoxins

**Importance of specific receptors on the brush border membrane of the mid-gut of target insects**

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To study the molecular basis of differences in the insecticidal spectrum of *Bacillus thuringiensis* δ-endotoxins, we have performed binding studies with three δ-endotoxins on membrane preparations from larval insect mid-gut. Conditions for a standard binding assay were established through a detailed study of the binding of 125I-labeled Bt2 toxin, a recombinant *B. thuringiensis* δ-endotoxin, to brush border membrane vesicles of *Manduca sexta*.

The toxins tested (Bt2, Bt3 and Bt73 toxins) are about equally toxic to *M. sexta* but differ in their toxicity against *Heliothis virescens*.

Equilibrium binding studies revealed saturable, high-affinity binding sites on brush border membrane vesicles of *M. sexta* and *H. virescens*. While the affinity of the three toxins was not significantly different on *H. virescens* vesicles, marked differences in binding site concentration were measured which reflected the differences in *in vivo* toxicity.

Competition experiments revealed heterogeneity in binding sites. For *H. virescens*, a three-site model was proposed. In *M. sexta*, one population of binding sites is shared by all three toxins, while another is only recognized by Bt3 toxin. Several other toxins, non-toxic or much less toxic to *M. sexta* than Bt2 toxin, did not or only marginally displace binding of 125I-labeled Bt2 toxin in this insect. No saturable binding of this toxin was observed to membrane preparations from tissues of several non-susceptible organisms.

Together, these data provide new evidence that binding to a specific receptor on the membrane of gut epithelial cells is an important determinant with respect to differences in insecticidal spectrum of *B. thuringiensis* insecticidal crystal proteins.

*Bacillus thuringiensis* is a Gram-positive bacterium which produces crystalline protein inclusions during sporulation. Due to their insecticidal properties, these proteins, designated δ-endotoxins or insecticidal crystal proteins (ICPs), have gained considerable academic and industrial interest. *B. thuringiensis* strains toxic to lepidopteran [1], dipteran [2] and coleopteran [3] insects have been identified. Moreover, within the group toxic to Lepidoptera, the insecticidal spectrum of the strains may be significantly different [1, 4–6]. Lepidoptera-specific ICPs generally are 130–140-kDa prototoxins. When ingested by susceptible insects, they are dissolved in the intestinal lumen and proteolytically processed to active toxins of about 60 kDa [7]. The toxins cause lysis of mid-gut epithelial cells [8].

The molecular basis for the differences in insecticidal spectrum of *B. thuringiensis* strains is still incompletely understood. Both the pH [9] and proteases [9, 10] in the insect gut lumen have been suggested as important factors. Only recently, the role of specific receptors on the plasma membrane of gut epithelial cells of target insects has been investigated. A binding assay with 125I-labeled toxin was first described by Hofmann et al. [11] and it was subsequently demonstrated that the difference in the insecticidal spectrum of two toxins on the tobacco hornworm (*Manduca sexta*) and the large white butterfly (*Pieris brassicae*) was correlated with the presence of high affinity binding sites [12]. We have investigated whether the importance of receptors as a determinant of the insecticidal specificity applies as a more general rule.

First we have studied the binding of Bt2 toxin to membrane vesicles of *M. sexta* under different conditions of pH, temperature, NaCl concentration and divalent cations. The kinetic parameters of the toxin-receptor interaction were also characterized. We then studied the toxicity and binding of three toxins (Bt3, Bt2 and Bt73 toxin) to two target insects (*M. sexta* and the tobacco budworm *Heliothis virescens*). These toxins belong to the CryIA(a)-, CryIA(b)- and CryIA(e)-type ICP respectively, according to the classification of Höfte and Whiteley [13]. Here we demonstrate that activation of protoxin does not significantly change the activities against the two insects. Further, we show that differences in activity against *H. virescens* are not determined by differences in affinity of the toxins for a single receptor site, but correlate with differences in binding site concentration. A three site model was proposed. The evidence for the importance of specific receptors in determining the specificity of ICPs is further strengthened using other toxins and membrane preparations from other insects.

**MATERIALS AND METHODS**

**Purification and activation of recombinant Bacillus thuringiensis δ-endotoxins**

The *bt2* gene was cloned from *B. thuringiensis* var. *berliner* 1715. The 130-kDa protein (Bt2 protoxin) was expressed in *Escherichia coli* as described by Höfte et al. [14]. Purification
of the recombinant protoxin and trypsin activation were also described in [14]. Activated toxin was further purified according to the method described by Hofmann et al. [12].

From *B. thuringiensis* var. *atrazinum* HD-68 a δ-endotoxin gene was cloned which encodes a 133-kDa protein (Bt3 protoxin) with insecticidal activity against *M. sexta* and *H. virescens* [15]. A gene cloned from *B. thuringiensis* var. *antoniocidus* HD110 codes for a 135-kDa protoxin preferentially active against *Spodoptera lituralis* and *Mamestra brassicae* (Bt13 protoxin) (H. Höfte, unpublished data). From *B. thuringiensis* var. *tenreiroensis* a gene was cloned which encodes a δ-endotoxin of molecular mass 73 kDa exhibiting toxicity against coleopteran larvae (Bt13 protoxin) [16]. The Bt8 protoxin is a recombinant mosquitocidal δ-endotoxin with a molecular mass of 130 kDa. The gene encoding this toxin was cloned from *B. thuringiensis* var. *israelensis* [17]. All these protoxins and their toxic fragments were purified according to the methods described for Bt2 (proto)toxin in [14] and [12]. The activated and purified toxins are further referred to as Bt2, Bt3, Bt13, Bt15 and Bt8 toxin.

**Purification of δ-endotoxin from a B. thuringiensis culture**

It has been shown that *B. thuringiensis* var. *kurstaki* HD73 produces only one single ICP [18]. A culture of this strain was grown as described by Mahillon and Delcour [19]. The autoysed culture was spun down (20 min at 4500 rpm in a HB4 rotor) and washed with a buffer containing 20 mM Tris, 100 mM NaCl and 0.05% Triton X-100, pH 8. The final pellet was resuspended in this buffer (4 ml buffer for 100 ml culture). This solution was then layered onto a linear Urograffin gradient (60–70%) which was centrifuged in a SW 28 rotor for 100 min at 100000 x g. The final pellet was then treated according to the methods described for Bt2 (proto)toxin in [14] and [12]. The purified toxin is further referred to as the Bt73 toxin.

**Toxicity tests**

Toxicity assays were performed on first instar larvae. Samples were diluted in NaCl/P (8 mM Na₂HPO₄, 2 mM KH₂PO₄, 150 mM NaCl, pH 7.4) with 0.1% bovine serum albumin (BSA), layered on artificial diet and allowed to dry. Larvae were then placed on the diet. Details on bioassays on *M. sexta* and *H. virescens* are given in [14] and [15], respectively. Mortalities were scored after 5 days (*M. sexta*) or 6 days (*H. virescens*). These data were analyzed by means of probit analysis [20]. Probit analysis is the standard method for analysis of toxicity data. Mortality percentages are transformed to probit 5 or 50% mortality on this regression line.

**Iodination of δ-endotoxins**

Iodination of Bt2, Bt3 and Bt73 toxin was performed using the chloramine-T method [21]. 1 mCi Na¹²⁵I and 20 – 37.5 μg chloramine-T in NaCl/P, were added to 50 μg purified toxin. After gentle shaking for 60 s, the reaction was stopped by adding 53 μg potassium metabisulfite in H₂O. The whole mixture was loaded on a PD-10 Sephadex G-25 M gel-filtration column to remove free iodine. A subsequent run on a Biogel P-60 column was carried out in order to increase the purity. Alternatively, toxins were labeled using the Iodogen method.

Iodogen (Pierce) was dissolved in chloroform at 0.1 mg/ml. 100 μl of this solution was pipetted into a disposable glass vessel and dried under a stream of nitrogen gas. The vessel was rinsed with Tris buffer (20 mM Tris, 0.15 M NaCl, pH 8.65). 50 μg toxin (in Tris buffer) was incubated with 1 mCi Na¹²⁵I in the tube for 10 min. The reaction was then stopped by the addition of 1 M NaI (one quarter of the sample volume). The sample was immediately loaded onto a PD10 Sephadex G-25M column and later on on a Biogel P-60 column to remove free iodine and eventual degradation products.

**Determination of specific activity of iodinated toxin**

Specific activity of iodinated toxin samples was determined using a ‘sandwich’ ELISA technique according to Voller [22]. Primary antibody was a polyclonal antiserum raised against Bt2 toxin and the second antibody was monoclonal antibody 4D6 (unpublished results). The conjugate used was alkaline phosphatase coupled to anti-(mouse IgG) antibodies. The reaction intensity of a standard dilution series of unlabeled toxin and dilutions of the iodinated toxin sample (in NaCl/P/BSA) was measured. Linear regression calculations yielded the protein content of the radioactive toxin sample.

The samples with the highest specific activities were used in the binding assays. Specific activities were 59-400 Ci/mmol, 33000 Ci/mol and 19800 Ci/mol (on reference date) for Bt73 toxin (labeled according to the Iodogen procedure), Bt2 toxin (chloramine-T method) and Bt3 toxin (Iodogen method), respectively.

**Preparation of brush border vesicles**

Brush border membrane vesicles from *M. sexta* and *H. virescens* were prepared according to the method of Woltersberger et al. [23]. This is a differential centrifugation method which makes use of the higher density of negative electrostatic charges on luminal than on basolateral membranes to separate these fractions. Vesicles of larval midguts of *Locusta migratoria migratoriana* and *Periplaneta americana*, as well as vesicles from the small intestine of mouse were kindly provided by Koen Hendrickx (KUL, Leuven). These vesicles were prepared according to the same method with a minor modification. Instead of 24 mM MgCl₂, a buffer (300 mM mannitol, 5 mM EGTA and 17 mM Tris/HCl, pH 7.5) containing 24 mM MgCl₂ was used. A plasmalemma preparation from fetal liver of chicken and vesicles from pig kidney were prepared according to the method of Shiu et al. [24] and Biber et al. [25], respectively, and were kindly provided by A. Vanderpoten (KUL, Leuven).

**Binding assay**

Duplicate samples of¹²⁵I-labeled toxin, either alone or in combination with varying amounts of unlabeled toxin, were incubated at the appropriate temperature with brush border membrane vesicles in a total volume of 100 μl of a particular buffer. The concentration of vesicles is given in the legends. Different buffers were used: 10 mM Tris, 150 mM NaCl, pH 7.4, 8.0 or 8.9, 50 mM Na₂CO₃, 100 mM NaCl, pH 10.0 and 8 mM Na₂HPO₄, 2 mM KH₂PO₄, 150 mM NaCl, pH 7.4 (NaCl/P). In one experiment, the Na₂HPO₄ and KH₂PO₄ concentration was varied between 0 and 10 mM, while in another the NaCl concentration ranged from 0–30 mM. All
buffers contained 0.1% bovine serum albumin. The incubation temperature was 20°C unless otherwise mentioned. Ultrafiltration through Whatman GF/F glass fiber filters was used to separate bound from free toxin. Each filter was rapidly washed with 5 ml ice-cold buffer (NaCl/Pi/BSA). The radioactivity of the filter was measured in a γ counter (1275 Minigamma, LKB). Data points correspond to the average values of duplicates that differed by less than 10%. Binding data were analyzed using the LIGAND computer program [26]. This program calculates the bound concentration of ligand as a function of the total concentration of ligand, given the affinity (Kd) and the total concentration of receptors or binding site concentration (R). The computer adjusts the initial estimates for Kd, R, and non-specific binding until the model approximates the actual data points as closely as possible. It uses an exact mathematical model, derived from the first-order mass action law (a) and the conservation of mass equation for the receptor sites (b) and the ligands (c) of the system: (a) \( L_i + R_j \rightarrow L_i R_j \), with \( K_{ij} = B_{ij}/F_i E_j \); (b) \( R_j = E_j + \Sigma_i B_{ij} \); (c) \( L_i = F_i + \Sigma_j E_{ij} \). \( L_i \) is total concentration of ligand i, \( R_j \) is total concentration of receptor j, \( B_{ij} \) is concentration of ligand i bound to receptor j, \( F_i \) is free concentration of ligand i, and \( E_j \) is concentration of empty receptor j.

The program allows statistical evaluation of the influence of binding of certain changes in the binding assay conditions. It is possible to assess whether an apparent change in the binding curve is significant and, if so, which parameter has changed. The program then compares the goodness of fit of different models using the 'extra sum of squares' principle. The calculated F ratio is compared to the tabulated value for the F statistic at a certain probability level [26].

Association experiments were performed as follows. Vesicles (100 μg vesicle protein/ml) were incubated with 1.05 nM \( ^{125}I \)-labeled Bt2 toxin. At different time intervals, duplicate samples of 100 μl were withdrawn from the incubation mixture and filtered. Values for association rate constants were calculated using the integrated second-order rate equation [27].

\[
\ln \left[ \frac{LR_e(L_e - LR(t) LR/R_e)}{L_e(L_e - LR)} \right] = k_i t \left( \frac{L_i R_i}{LR_e} - \frac{L_e}{LR_e} \right).
\]

In Eqn (1) \( L_e \) is the total concentration of ligand, \( R_e \) the total concentration of binding sites, \( LR_e \) the concentration of ligand-receptor complex at equilibrium and \( LR(t) \) the concentration of ligand-receptor complex at time t. Non-specific binding, defined as the binding in the presence of 75 nM (20°C and 30°C) or 37.5 nM (4°C) unlabeled Bt2 toxin, was subtracted from total binding for the analysis of the data.

In order to study dissociation kinetics, the reaction was reversed when equilibrium was reached. The incubation mixture was either tenfold diluted in NaCl/Pi/BSA or an excess (75 nM) unlabeled Bt2 toxin was added.

In the former case, samples of 1 ml were removed from the mixture. The dissociation rate constant was calculated using the following equation [27].

\[
\ln \left[ LR(t)/LR_o \right] = -k_{-1} t.
\]

In this equation, \( LR_o \) is the concentration of ligand-receptor complex just prior to dilution or addition of excess unlabeled ligand and \( LR(t) \) is the concentration of ligand-receptor complex at time t after initiation of dissociation. As a part of the toxin tended to be irreversibly bound (see results), this part was subtracted from \( LR(t) \) and \( LR_o \).

**Autoradiography**

Labeled toxins were incubated for 30 min with \( M.\ sexta \) or \( H.\ virescens \) vesicles at room temperature. The samples were spun down for 15 min at 14000 rpm in a Heraeus microcentrifuge. The pellet was resuspended in NaCl/Pi/BSA and again centrifuged. The final pellet, the first supernatant and toxin not incubated with vesicles were separated on SDS/PAGE (10% acrylamide) [28]. The dried gel was exposed to Fuji RX Safety film for 1–7 days.

**Determination of protein concentration**

Protein concentration of purified Bt2, Bt3, Bt73 and Bt15 toxins were calculated from the absorbance at 280 nm (measured with a Uvikon 810 P, Kontron Instruments spectrophotometer). The protein content of solutions of other toxins and of brush border membrane vesicles was measured according to Bradford [29].

**RESULTS**

**Effect of different parameters on binding**

The influence of different parameters on the binding of \( ^{125}I \)-labeled Bt2 toxin to \( M.\ sexta \) vesicles was studied. The dissociation constant (Kd) and the binding site concentration (R) slightly decreased with increasing temperature (Table 1). No gross changes in Kd or R occurred in the pH range 7.4–10.0, though binding was somewhat reduced at pH 10.0 (Table 1). Binding was very clearly influenced by the concentration of NaCl (Table 1). At 0 and 25 mM NaCl, no saturable binding was observed. At 75 mM NaCl, there was saturable binding, though the non-specific binding was rather high (16%). Increasing the NaCl concentration to 0.15 M and 0.3 M NaCl had no major effects on Kd and R, but lowered non-specific binding. Substitution of the K+ ions with Na+ ions or vice versa, or addition of 5 mM EGTA, 10 mM Mg or Ca ions did not significantly influence Bt2-toxin binding (data not shown). On the basis of these results we selected the following buffer for further binding experiments: 8 mM Na3HPO4, 2 mM KH2PO4, 150 mM NaCl, pH 7.4, with 0.1% bovine serum albumin.

**Kinetics of binding**

The time-dependent association of \( ^{125}I \)-labeled Bt2 toxin to \( M.\ sexta \) vesicles was studied at 4°C, 20°C and 30°C (Fig. 1A). A straight line is obtained when these data are plotted according to the integrated form of the second-order rate equation, suggesting a simple bimolecular reaction (Fig. 1A, inset). Assuming a binding site concentration of 3.4 pmol/mg vesicle protein, the mean kinetic association constant was calculated to be 0.77(± 0.15)×108 M−1 min−1, 2.4(± 0.42)×108 M−1 min−1 and 2.9(± 0.55)×108 M−1 min−1 at 4°C, 20°C and 30°C, respectively (means of four independent experiments).

The dissociation process was biphasic at 20°C and 30°C, with a major fraction of the toxin irreversibly bound (Fig. 1B). At 4°C virtually no dissociation was observed. Due to the minor fraction of reversibly bound toxin, only estimates could be obtained for the dissociation rate constant of the 'fast' component of the dissociation process: 0.072 ± 0.019 min−1 at 20°C and 0.068 ± 0.027 min−1 at 30°C (means of three experiments). The Kd values estimated on the basis of these
Values for the dissociation constant \( K_d \) of binding sites \( (R) \) and non-specific binding were calculated from competition experiments. Brush border membrane vesicles (100 μg vesicle protein/ml) were incubated with 1.05 nM \(^{125}\text{I}\)-labeled Bt2 toxin in the presence of increasing concentrations of unlabeled Bt2 toxin. Samples were filtered through Whatman GF/F filters and washed with 5 ml cold buffer (NaCl/P/BSA). Data were analyzed with the I.GAND computer program. Not determined, nd. For each of the three groups of experiments (temperature, pH and NaCl concentration), a statistical analysis was performed. NSB, non-specific binding. Values for \( R \), values obtained are significantly different for each binding measurement \( (P < 0.01) \). The values obtained at pH 7.4, 8.0 and 8.9 are not significantly different for each binding measurement \( (P < 0.01) \). The values obtained at 75 mM and 300 mM NaCl are not significantly different for each binding measurement \( (P < 0.01) \).

### Table 2. Toxicity of B. thuringiensis \( \delta \)-endotoxins on M. sexta and H. virescens

<table>
<thead>
<tr>
<th>Toxin</th>
<th>M. sexta</th>
<th>H. virescens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( LC_{50} )</td>
<td>( CI_{95} )</td>
</tr>
<tr>
<td></td>
<td>ng/cm²</td>
<td></td>
</tr>
<tr>
<td>Bt2 toxin</td>
<td>20</td>
<td>15 - 28</td>
</tr>
<tr>
<td>Bt3 toxin</td>
<td>20</td>
<td>15 - 29</td>
</tr>
<tr>
<td>Bt73 toxin</td>
<td>9</td>
<td>6 - 12</td>
</tr>
<tr>
<td>(^{125}\text{I}-)Bt2-toxin</td>
<td>20</td>
<td>15 - 27</td>
</tr>
<tr>
<td>(^{125}\text{I}-)Bt3-toxin</td>
<td>20</td>
<td>14 - 29</td>
</tr>
<tr>
<td>(^{125}\text{I}-)Bt73-toxin</td>
<td>8</td>
<td>6 - 11</td>
</tr>
<tr>
<td>Bt15 toxin</td>
<td>110</td>
<td>76 - 163</td>
</tr>
<tr>
<td>Bt13 toxin</td>
<td>&gt; 128</td>
<td></td>
</tr>
<tr>
<td>Bt8 toxin</td>
<td>&gt; 128</td>
<td></td>
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</tbody>
</table>

### Toxicity of activated ICPs

We have used the Bt3, Bt2 and Bt73 toxin as representatives of the CryIA(b) and CryIA(c) class of \( B. thuringiensis \( \delta \)-endotoxins respectively (Table 2). \( M. sexta \) was equally susceptible to Bt2 toxin as to Bt3 toxin. The toxicity of Bt73 toxin was slightly higher. The \( LC_{50} \) values were 20 ng/cm², 20 ng/cm² and 9 ng/cm² respectively. In contrast, these \( \delta \)-endotoxins exhibited clear differences in toxicity when tested against \( H. virescens \) larvae. Bt73 toxin was very active on this insect \( (LC_{50} = 2 \text{ ng/cm}^2) \) while the activity of Bt2 and Bt3 toxin, as expressed by the \( LC_{50} \) value, was respectively 3.5 and 75 times lower. This differential spectrum was previously reported at the level of the protoxins [15].

### Binding of activated ICPs to insect membranes

The purified \(^{125}\text{I}\)-labeled Bt2, Bt3 and Bt73 toxin showed a single 60-kDa band on autoradiography (Fig. 2, lanes 1, 4 and 7). All three toxins fully retained their toxicity after a
reaction with $^{125}$I (see Table 2). After a 30 min incubation, no degradation was observed of either free or bound $^{125}$I-labeled toxin with both $M.\ sexta$ (Fig. 2, lanes 3, 6, 9 and lanes 2, 5, 8, respectively) and $H.\ virescens$ vesicles (data not shown).

As a necessary control experiment, binding to tissues of non-susceptible organisms [30–32] (S. Jansens, unpublished data) was studied. Saturable binding of $^{125}$I-labeled B2 toxin to brush border membrane vesicles of larval mid-guts of L. migratoria migratorioides and P. americana, to vesicles of the small intestine of mouse and of pig kidney and to a plasmalemma preparation of fetal liver of chicken was negligible. None of these preparations bound more than 1% of $^{125}$I-labeled B2 toxin, even at 1000 µg vesicle protein/ml. In contrast, about 30% of the toxin was saturably bound to $M.\ sexta$ membrane vesicles at 100 µg vesicle protein/ml.

Displacement studies (incubation with increasing amounts of labeled toxin) revealed high-affinity saturable binding for all three toxins on membrane preparations of both $M.\ sexta$ and $H.\ virescens$ (Table 3). LIGAND analysis indicated that for all three toxins a one-site model was compatible with these binding data. On $H.\ virescens$ vesicles, the mean affinities (three replicates) of the three toxins were not significantly different (Duncan test). On $M.\ sexta$ vesicles, the mean affinity of B3 toxin significantly ($P < 0.05$, Duncan test) differed from the mean affinities of the other toxins.

The mean concentration of binding sites for B3 toxin was higher than for B2 and B373 toxin in $M.\ sexta$ ($R_i = 9.8$ compared to 3.4 pmol/mg and 4.0 pmol/mg vesicle protein). This difference was statistically significant ($P < 0.05$, Duncan test).

Considerable differences in the concentration of binding sites could also be demonstrated for $H.\ virescens$ membrane vesicles. For B373 toxin, a mean $R_i$ value of 19.5 pmol/mg vesicle protein was calculated, which was approximately 2 and 6 times higher than the mean $R_i$ for B2 and B3 toxins, respectively. Only the difference between the means for B3 and B373 toxin was significant ($P < 0.05$, Duncan test). However, the large standard deviations (see Table 3) are largely due to between-experiment variation in $R_i$. In two of the three experiments, all three toxins were studied simultaneously and the ratios of binding site concentrations for B373/B2/B3 toxins were then quite similar: 6.0:3.5:1 and 7.2:3.2:1. Statistical analysis of both of these simultaneous experiments (thus excluding between-experiment variation) with the LIGAND program, indicated that all of these differences were highly significant ($P < 0.01$).

Results of homologous competition experiments (competition of unlabeled toxin for the labeled form) (Table 4) were in good agreement with those of the displacement experiments. In most cases, the affinity of unlabeled toxin was about twice the affinity of the iodinated toxin (compare with Table 3). Experiments on $M.\ sexta$ confirmed the binding site concentrations obtained from displacement studies. The difference in $R_i$ between B3 toxin on one hand and B2 and B373 toxins on the other hand was again found, although less pronounced. Experiments on $H.\ virescens$ indicated even more pronounced differences in $R_i$ compared to the data of the displacement studies. For B373 toxin, a mean value of 62.3 pmol/mg vesicle protein was obtained, while the binding

<table>
<thead>
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<th>Insect</th>
<th>Toxin</th>
<th>$R_i$</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M.\ sexta$</td>
<td>Bt2</td>
<td>3.4 (0.3)</td>
<td>0.4 (0.04)</td>
</tr>
<tr>
<td>$M.\ sexta$</td>
<td>Bt3</td>
<td>9.8 (1.3)</td>
<td>1.5 (0.36)</td>
</tr>
<tr>
<td>$M.\ sexta$</td>
<td>Bt373</td>
<td>4.0 (0.2)</td>
<td>0.6 (0.03)</td>
</tr>
<tr>
<td>$H.\ virescens$</td>
<td>Bt2</td>
<td>9.7 (3.4)</td>
<td>0.6 (0.06)</td>
</tr>
<tr>
<td>$H.\ virescens$</td>
<td>Bt3</td>
<td>3.7 (0.5)</td>
<td>1.2 (0.52)</td>
</tr>
<tr>
<td>$H.\ virescens$</td>
<td>Bt373</td>
<td>19.5 (8.1)</td>
<td>0.8 (0.22)</td>
</tr>
</tbody>
</table>

Table 3. Concentration of binding sites and equilibrium dissociation constant of Bt2, Bt3 and Bt373 toxin on brush border membrane vesicles of $M.\ sexta$ and $H.\ virescens$, as calculated from displacement experiments

Increasing amounts of $^{125}$I-labeled Bt2, Bt3 and Bt373 toxins were incubated with vesicles ($M.\ sexta$ vesicles; 100 µg vesicle protein/ml; $H.\ virescens$ vesicles, 50, 100 and 150 µg vesicle protein/ml in experiments with, respectively, Bt3, Bt2 and Bt3 toxin) for 30 min at room temperature. For each toxin, 12 concentrations were used in the following range: 0.15–18 nM $^{125}$I-labeled B2 toxin; 0.3–51.7 nM $^{125}$I-labeled B2 toxin; 0.09–35.5 nM $^{125}$I-labeled B373 toxin. Samples were processed as described in the legend to Table 1. Values given are the mean of three repeat experiments. Standard deviations are given between brackets.

<table>
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<th>Toxin</th>
<th>$R_i$</th>
<th>$K_d$</th>
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<tbody>
<tr>
<td>$M.\ sexta$</td>
<td>Bt2</td>
<td>7.9 (0.28)</td>
<td>0.2 (0.04)</td>
</tr>
<tr>
<td>$M.\ sexta$</td>
<td>Bt3</td>
<td>9.9 (1.5)</td>
<td>1.1 (0.3)</td>
</tr>
<tr>
<td>$M.\ sexta$</td>
<td>Bt373</td>
<td>6.3 (1.4)</td>
<td>0.2 (0.01)</td>
</tr>
<tr>
<td>$H.\ virescens$</td>
<td>Bt2</td>
<td>21 (15.7)</td>
<td>0.4 (0.04)</td>
</tr>
<tr>
<td>$H.\ virescens$</td>
<td>Bt3</td>
<td>3.7 (0.6)</td>
<td>0.8 (0.7)</td>
</tr>
<tr>
<td>$H.\ virescens$</td>
<td>Bt373</td>
<td>62.3 (6.9)</td>
<td>0.4 (0.14)</td>
</tr>
</tbody>
</table>

Table 4. Concentration of binding sites and equilibrium dissociation constant of Bt2, Bt3 and Bt373 toxin on brush border membrane vesicles of $M.\ sexta$ and $H.\ virescens$, as calculated from homologous competition experiments

Brush border membrane vesicles (for concentrations see Table 3) were incubated with labeled toxin (1.05 nM $^{125}$I-labeled B2 toxin; 0.8 nM $^{125}$I-labeled B3 toxin; 1.05 nM $^{125}$I-labeled B373 toxin) in the presence of increasing concentrations of Bt2, Bt3, or Bt373 toxin, respectively. These duplicate samples were processed as described in the legend of Table 1. Each value given is the mean value of two or more independent repeat experiments. Standard deviations are indicated between brackets.
Fig. 3. Binding of $^{125}$I-labeled toxins to *M. sexta* (*A, B, C*) and *H. virescens* (*D, E, F*) brush border membrane vesicles. Vesicles (for concentrations see legend to Table 3) were incubated with labeled toxin (*A* and *D*, 1.05 nM $^{125}$I-labeled Bt2 toxin; *B* and *E*, 0.8 nM $^{125}$I-labeled Bt3 toxin; *C* and *F*, 1.05 nM $^{125}$I-labeled Bt73 toxin) in the presence of increasing concentrations of Bt2 toxin (*•*), Bt3 toxin (*○*) or Bt73 toxin (*△*). Binding is expressed as percentage of the amount bound upon incubation with labeled toxin alone. On *M. sexta* vesicles these amounts were 1820 cpm, 601 cpm and 2383 cpm and on *H. virescens* vesicles 1775 cpm, 472 cpm and 6608 cpm for $^{125}$I-labeled Bt2, Bt3 and Bt73 toxins, respectively. Non-specific binding was not subtracted. Data were analyzed with the LIGAND computer program. Each point is the mean of a duplicate sample.

In short, the above experiments show that the binding site concentration, but not the affinity, was different for the three toxins in the case of *H. virescens* membrane vesicles. On *M. sexta* vesicles, Bt3 toxin had a lower affinity and higher binding site concentration than the other toxins.

**Heterogeneity between binding sites**

For each labeled ligand, competition experiments with the three unlabeled ligands were performed in order to investigate the relationship between the binding sites for the three toxins. The three toxins displaced all $^{125}$I-labeled Bt2 and Bt73 toxins from *M. sexta* membrane vesicles in the same nanomolar concentration range (Fig. 3A and C). In these experiments, the competition curve for Bt3 toxin was slightly shifted to the right compared to the curves for the other toxins. This confirms the lower affinity of Bt3 toxin. Competition experiments with labeled Bt3 toxin demonstrated that Bt2 and Bt73 toxin could only displace about 60% to 70% of the bound Bt3 toxin relative to unlabeled Bt3 toxin (Fig. 3B). This confirms the higher concentration of binding sites for the Bt3 toxin.
The three toxins competed with comparable affinity for binding of labeled Bt3 toxin on vesicles of *H. virescens* (Fig. 3E). The binding of 125I-labeled Bt2 toxin was entirely saturable with Bt2 and Bt73 toxin. However, Bt3 toxin could only partially saturate 125I-labeled Bt2-toxin-binding sites (Fig. 3D). Bt2 toxin competed, with about the same affinity as the Bt73 toxin, for the major part of bound 125I-labeled Bt73 toxin (Fig. 3F). However, there was a small part of saturably bound Bt73 toxin which could not be displaced by Bt2 toxin. This small difference was consistent throughout the repeat experiments. High concentrations of Bt3 toxin were required for only marginal competition with labeled Bt73 toxin.

Competitive binding of other toxins

Several other toxins were compared with Bt2 toxin for their ability to displace bound 125I-labeled Bt2-toxin from *M. sexta* vesicles. Bt5 toxin is a recombinant δ-endotoxin active against *S. littoralis* and *M. brassicae* but exhibiting, in comparison with Bt2 toxin, only poor toxicity against *M. sexta* (Table 1). In the absence of competitor, 56% of labeled Bt2 toxin was bound to the vesicles. In the presence of 72 nM and 720 nM Bt5 toxin, the percentage bound was 54% and 50%, respectively. Thus, Bt5 toxin does not recognize the binding site of Bt2 toxin. Bt5 toxin, a coleopteran-specific toxin, and Bt8 toxin, a δ-endotoxin with high mosquitocidal potency, have no detectable toxicity against *M. sexta* at concentrations up to 128 ng/cm² (Table 2). Bt13 toxin did not affect the binding of 125I-labeled Bt2 toxin. Whereas Bt8 protoxin did not compete for binding of Bt2 toxin [12], the trypsin-activated protein (Bt8 eight), had a limited effect: in the presence of 7.2 nM and 72 nM Bt8 toxin, respectively, 46% and 38% binding of labeled Bt2 toxin was observed.

**DISCUSSION**

In this report we investigated the molecular basis of differences in insecticidal spectrum of three *B. thuringiensis* δ-endotoxins. We found it essential to first study the binding of a model ICP (Bt2 toxin) to brush border membrane vesicles of *M. sexta* in more detail. The influence of variations in pH, temperature, NaCl concentration and divalent cations was determined and on the basis of these data, standard conditions for the binding assay were selected. These conditions, under which binding is near-optimal, are similar to the conditions prevailing in the midgut of *M. sexta*. The mid-gut lumen of most lepidopteran larvae has been reported to be very alkaline [33]. Delello et al. [34] reported an average pH value of the mid-gut of *M. sexta* of 9.3. Accordingly, one might expect that binding would be optimal at pH values of 9.0—10.0. Whereas binding was not dependent on the pH between pH 7.4 and pH 8.9, the decreased binding at pH 10.0 which we observed is probably due to instability of the vesicles at this high pH and does not necessarily reflect suboptimal binding. The mid-gut lumen of *M. sexta* was shown to contain no detectable level of Na⁺ ions but a high concentration of K⁺ ions (180 mM) [35]. While a high K⁺/Na⁺ ratio is characteristic for the mid-gut, we have demonstrated that these ions can be interchanged in the binding assay without significantly affecting the binding, provided that their total concentration is at least 75 mM. Further, we have shown that Mg²⁺ or Ca²⁺ ions are not necessary for binding. Such a result was anticipated, since Dow et al. [35] did not detect Mg²⁺ or Ca²⁺ ions in the gut lumen of *M. sexta*.

For all the parameters, broad optima were observed. This is an important element to support conclusions drawn from binding studies performed with different toxin/insect combinations. Observed differences in binding are thus likely to reflect the intrinsic characteristics of each toxin-membrane interaction. It is unlikely that the data are influenced by deviations from optimal binding conditions for each such combination.

Association of 125I-labeled Bt2 toxin at 4°C, 20°C and 30°C apparently obeys simple bimolecular kinetics. The biphasic dissociation process however suggests a more complex reaction, probably a two-step binding mechanism. The apparent irreversibility could be due to a conformational change of the toxin-receptor complex or a (partial) integration of the toxin into the membrane. The existence of a biphasic dissociation process poses theoretical problems for the analysis of the data using simple equilibrium equations. However, several authors have employed such equations in similar cases and found this approach to give a good approximation to their data [36—38].

We selected the Bt2, Bt3 and Bt73 toxin to study the molecular basis of specificity of *B. thuringiensis* ICPs. The three toxins exhibited only limited differences in larvicidal activity on *M. sexta*. However, the difference in toxicity to *H. virescens* was more pronounced: on the basis of the LC₅₀p, Bt3 toxin was about 20 times less active than Bt2 toxin, which in turn was about 3 times less active than Bt73 toxin. We previously observed a similar toxicity pattern for the corresponding protoxins [15]. This demonstrates that in these insects activation of protoxin is not a key factor with respect to the differential toxicity of these ICPs. Lecadet and Martouret [4] also demonstrated that activation, either by trypsin or by gut proteases from either *S. littoralis* or *P. brassicae*, did not significantly alter the toxicity of ICPs of two strains. In contrast, Haider et al. [10] showed that activation of protoxin from *B. thuringiensis* var. *colmeri* by gut proteases of *P. brassicae* or *Aedes aegypti* resulted in a different insecticidal spectrum of these preparations. Since 125I-labeled toxins were found to be stable in the presence of vesicles from both *H. virescens* and *M. sexta* (unpublished data), membrane associated proteases can also be excluded as a possible determinant of differences in insecticidal spectrum.

We observed high-affinity saturable binding for the three toxins to brush border membrane vesicles of the susceptible insects, *M. sexta* and *H. virescens*. In contrast, there was no significant saturable binding to membrane preparations from a number of non-susceptible organisms.

The affinities of the three toxins for *H. virescens* vesicles were not significantly different, indicating that the differences in toxicity are not due to differences in affinity for a single binding site in the mid-gut. In contrast, considerable differences in the concentration of binding sites were observed, reflecting the differences in toxicity. Thus, in *H. virescens*, a correlation (though not a linear one) was found between toxicity and the binding site concentration. Competition studies revealed a complex relationship between the binding sites for the three toxins in *H. virescens*. In our opinion, the most plausible hypothesis to explain this heterogeneity in binding sites consists of a three-site model. One population of binding sites binds all three toxins. A second population binds Bt2 and Bt73 toxins but not Bt3 toxin, while a third population is only accessible for the most toxic δ-endotoxin, Bt3 toxin. This model may still underestimate the actual complexity of toxin-membrane interactions. Indeed, while all the data are qualitatively in good agreement with this model, there are
some discrepancies at a quantitative level. According to the proposed model and the results of the displacements experiments, Bt2 toxin should displace less $^{125}$I-labeled Bt73 toxin than actually observed. Furthermore, Bt3 toxin should compete for about one-third of the binding sites of Bt2 toxin with a $K_d$ similar to that of Bt2 toxin. However, no appreciable competition with such affinity was found. At present, we do not understand the reason for these discrepancies. Possibly only a minor fraction of the Bt2-toxin-binding sites was accessible for Bt3 toxin. This fraction may have been so small that its saturation could not be clearly seen in competition curves. The decrease in binding of labeled Bt2 toxin at concentrations of Bt3 toxin higher than 10 nM could be due to the saturation of the other population of Bt2-toxin-binding sites for which Bt3 toxin would then compete with a much lower affinity (more than 100-fold). In *M. sexta* vesicles, the binding site concentration for Bt3 toxin was higher than that for the other toxins. This is not reflected in a higher toxicity of Bt3 toxin. It should however be noted that the binding affinity of the latter toxin is lower than the affinity of Bt2 and Bt73 toxin.

We suggest that the lower affinity compensates to a certain extent the higher concentration of binding sites in vivo. The higher binding site concentration for Bt3 toxin in *M. sexta* was confirmed by heterologous competition experiments. Thus, it appears that there is one population of sites recognized by all three toxins, whereas Bt3 toxin recognizes additional sites.

Toxins with no detectable or only low activity against *M. sexta*, did not or only marginally compete for the sites occupied by $^{125}$I-labeled Bt2 toxin. This observation provides additional evidence for the specific nature of the observed binding.

The toxins extensively tested here all belong to a related family of *B. thuringiensis* crystal proteins. Different authors demonstrated that the active moiety of the protoxin is located in its N-terminal half [14, 39–42]. In the case of Bt2 toxin, this fragment was shown to be located between amino acids 29 and 607 [14]. The CryIA(a)-, CryIA(b)-, and CryIA(c)-type crystal proteins show a high level of sequence similarity in the N-terminal part of this toxic fragment (up to position 270) but considerable differences in sequences occur in the other part (between amino acids 270 and 600) [42, 43]. Our data strongly suggest that these differences play an important role in determining the binding characteristics and hence the insecticidal spectrum of these toxic types.

In an earlier report, we demonstrated a correlation between binding and toxicity of two $\delta$-endotoxins [12]. The results presented here provide additional evidence for such a correlation. Furthermore, they indicate heterogeneity in binding sites in *H. virescens* and *M. sexta* membrane vesicles. Binding site heterogeneity appears as a more general characteristic of ICP-membrane interaction, since it was also described by Hofmann et al. [12]. These authors demonstrated that two $\delta$-endotoxins, both toxic to *P. brassicae*, occupied distinct populations of binding sites on brush border membrane vesicles of this insect. Binding site heterogeneity could have practical importance. The application of an ICP with different binding sites in the mid-gut of target insects or the application of a mixture of ICPs which recognize different binding sites could diminish the chance of the development of resistance to ICPs in these insects.

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