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A Binding Site for *Bacillus thuringiensis* Cry1Ab Toxin Is Lost during Larval Development in Two Forest Pests

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The insecticidal activity and receptor binding properties of *Bacillus thuringiensis* Cry1A toxins towards the forest pests *Thaumetopoea pityocampa* (processionary moth) and *Lymantria monacha* (nun moth) were investigated. Cry1Aa, Cry1Ab, and Cry1Ac were highly toxic (corresponding 50% lethal concentration values: 956, 895, and 379 pg/μl, respectively) to first-instar *T. pityocampa* larvae. During larval development, Cry1Ab and Cry1Ac toxicity decreased with increasing age, although the loss of activity was more pronounced for Cry1Ab. Binding assays with ^{125}I-labelled Cry1Ab and brush border membrane vesicles from *T. pityocampa* first- and last-instar larvae detected a remarkable decrease in the overall Cry1Ab binding affinity in last-instar larvae, although saturable Cry1Ab binding to both instars was observed. Homologous competition experiments demonstrated the loss of one of the two Cry1Ab high-affinity binding sites detected in first-instar larvae. Growth inhibition assays with sublethal doses of Cry1Aa, Cry1Ab, and Cry1Ac in *L. monacha* showed that all three toxins were able to delay molting from second instar to third instar. Specific saturable binding of Cry1Ab was detected only in first- and second-instar larvae. Cry1Ab binding was not detected in last-instar larvae, although specific binding of Cry1Aa and Cry1Ac was observed. These results demonstrate a loss of Cry1Ab binding sites during development on the midgut epithelium of *T. pityocampa* and *L. monacha*, correlating in *T. pityocampa* with a decrease in Cry1Ab toxicity with increasing age.

The discovery of *Bacillus thuringiensis* has had the greatest impact on the use of biopesticides in forestry, as well as in crop systems and stored products. *B. thuringiensis* is a bacterium that produces proteinaceous insecticidal toxins in the form of inclusion bodies or crystals at sporulation. Each toxin specifically recognizes and binds to receptors in the insect gut, forming membrane pores that disrupt the selective permeability of the cells and eventually cause lysis of epithelial cells and insect death (5, 11, 22, 36).

Binding of toxins to midgut receptors is a key step in the mode of action of *B. thuringiensis* toxins (5, 6, 14, 15, 36, 37). Specific binding of lepidopteran-active toxins has been demonstrated by incubating midgut brush border membrane vesicles (BBMVs) (14, 15, 36, 37), tissue sections (5, 6), and BBMV protein blots (10, 26) with radiolabelled or biotin-labelled toxins. These studies aided in the characterization of the receptor system of many insect pests and identified several toxin-binding proteins (7, 12, 20, 21, 23, 30, 32).

The use of *B. thuringiensis* as a biopesticide has proven to be a viable alternative to chemical insecticides (25). There has been a major increase in the proportion of areas treated with *B. thuringiensis* in North America, particularly in Eastern Canada (33, 34), and in Eastern Europe, at the expense of chemical insecticides such as fenitrothion (Canada) and diflubenzuron and pyrethroids (Eastern Europe). This trend is expected to continue in the future, with at least 50% of forest areas being treated with microbial insecticides, of which *B. thuringiensis* will remain dominant (8). The environmental benefit of their increased use is their specificity and the possibility to use them in both rural and urban woods (25).

This paper analyzes the toxicity and binding of *B. thuringiensis* toxins in two important forest pests, *Thaumetopoea pityocampa* and *Lymantria monacha*, in an attempt to optimize the use of this microbial pathogen to control the two forest pests in the field.

T. pityocampa, processionary moth, is an important pine defoliator that represents a major endemic pest in Southern Europe. The larvae are covered with urticating hairs that can cause serious skin irritation in those living in close proximity to infested trees, and there is, thus, considerable public pressure to manage this moth population.

The nun moth, *L. monacha*, is one of the most severe insect pest species of European conifers, although this insect can also affect other host genera, such as *Quercus*, *Fagus*, and *Betula*, and even herbs. This is an epidemic pest distributed throughout Europe and parts of Asia, south of 60° latitude, thus occurring from Portugal to Japan.

We have previously demonstrated that Cry1Aa, Cry1Ab, and Cry1Ac specifically bound to the midgut brush border membrane of second-instar *T. pityocampa* larvae and that all three toxins compete for binding (28). In the present paper, we correlate the decrease in Cry1Ab toxicity during development with the loss of a binding site for this toxin in last-instar larvae. Similarly, in *L. monacha* we have found that binding of Cry1Ab to the larval midgut membrane was lost during the larval development of the insect.

MATERIALS AND METHODS

Biological material. *T. pityocampa* S. and *L. monacha* L. larvae were used in all experiments. *T. pityocampa* larvae were collected from natural populations in Burjassot (Valencia, Spain). Larvae were fed on pine needles (*Pinus sylvestris*) at room temperature. *L. monacha* larvae were reared from egg masses collected from Orihuela del Tremedal (Teruel, Spain). Eggs were stored at 4°C until needed and then incubated at 25°C for hatching. At eclosion, larvae were transferred to petri dishes and reared, until third instar, on an artificial diet, according to the method of Grijpma et al. (13), except that aureomycin was omitted due to incompatibility with *B. thuringiensis* treatments (27). From third instar until pupation, larvae were fed on *Pinus halepensis* needles. Rearing conditions were as follows: 25°C, 70% relative humidity, and a 16-h–8-h (light-dark) photoperiod.

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Insect toxicity assays. Bioassays were carried out to determine the sensitivity of first-instar *T. pityocampa* larvae to Cry1A-type *B. thuringiensis* trypsin-activated toxins. Trypsin digestion and toxin purification were performed by the method of Höfte et al. (16). Each bioassay consisted of five doses of the corresponding activated crystal toxins (Cry1Aa, Cry1Ab, and Cry1Ac) prepared in phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4) containing 0.1% bovine serum albumin (BSA) and a control of PBS (pH 7.4)–0.1% BSA. Fresh *P. sylvestris* needles were dipped into this suspension and allowed to air dry. Three groups of 20 larvae (1 day old) were placed on the coated pine needles for 4 days, after which the coated needles were replaced with fresh, untreated needles. Mortality was recorded 4 days later. Toxicity data were evaluated by probit analysis using the Polo PC program (LeOra Software, Berkeley, Calif.). Fifty percent lethal concentration (LC_{50}) measurements refer to the concentration of crystal protein which, when applied uniformly on the needles, produces 50% mortality.

Toxicities of Cry1Ab (1 and 10 ng/μl) and of Cry1Ac (0.4 and 4 ng/μl) were also tested on third-instar *T. pityocampa* larvae as described above.

To study sublethal effects of *B. thuringiensis* Cry1A-type toxins on *L. monacha* development, freshly prepared artificial diet was dispensed in wells with 2 cm^2 of surface area (Costar 24-well cluster plate), and dilutions of the corresponding toxin (50 μl; prepared in PBS [pH 7.4]–0.1% BSA) were uniformly applied over the food surface in each well and allowed to dry. Single larvae in the first day of the second instar were placed in each well; 20 larvae were used per dilution. Three dilutions of each trypsin-activated toxin (Cry1Aa, Cry1Ab, and Cry1Ac), corresponding to 2, 20, and 200 ng/cm^2, were analyzed. After five days of toxin exposure, the percentage of larvae reaching third instar was scored. Experiments were duplicated, and controls with PBS were also included.

Binding to midgut tissue sections. The midgut tissue preparation, sectioning, binding, and immunocytochemical staining were performed according to the method of Bravo et al. (5). Experiments involving binding of Cry1Ab and Cry1Ac trypsin-activated toxins to *T. pityocampa* tissue sections from first- and third-instar larvae were carried out as follows. Tissue sections were incubated with the appropriate toxin for 1 h by using 0.3 ml of a mixture of 10 μg of toxin per ml of TST buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM sodium ethylmercurisalicylate, 0.1% [vol/vol] Triton X-100, pH 7.6). Immunolocalization of toxins was performed by incubating tissue sections overnight with 0.3 ml of the monoclonal antibody 4D6 (Plant Genetic Systems, Ghent, Belgium) (0.1 μg/ml in TST buffer). Unbound antibody was rinsed with TST buffer, and an alkaline phosphatase-coupled secondary antibody was used to detect the binding. Color development was obtained by incubation in 0.5 ml of BCIP-nitroblue tetrazolium (NBT) solution (8 mM 5-bromo-4-chloro-3-indolyl phosphate and 9 mM 4-NBT chloride in 5 mM $MgCl_2$, 100 mM Tris, 100 mM NaCl, pH 9.5) for 10 min. Color development was stopped by lowering the pH with glacial acetic acid-water (1:10, vol/vol). Slices were dehydrated by successive incubations in 70% and 100% ethanol and 100% xylol. Finally, tissue sections were covered with Entellan mounting medium (Merck, Darmstadt, Germany) and photographed.

Preparation of BBMVs. BBMVs were prepared according to the method of Wolfersberger et al. (40) from *T. pityocampa* first- and early second-instar larvae, *T. pityocampa* last-instar larvae, *L. monacha* first- and early second-instar larvae, and *L. monacha* last-instar larvae. All BBMV preparations were from whole larvae of the corresponding instar, except for *L. monacha* last-instar larvae, from which dissected midguts were used. The final pellet was resuspended in ice-cold MET buffer (0.3 M mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5), immediately frozen, and stored at −80°C.

The protein concentration of BBMVs was measured by Bradford's procedure (4) with a Bio-Rad kit (Richmond, Calif.), with BSA as the standard.

Labelling of toxins. Cry1Aa, Cry1Ab, and Cry1Ac trypsin-activated toxins were iodinated by the chloramine-T method (17) as described by Van Rie et al. (36). The specific radioactivities of iodinated toxins, determined by a sandwich enzyme-linked immunosorbent assay technique, were 5 mCi/mg for Cry1Aa, 36 mCi/mg for Cry1Ab, and 4 mCi/mg for Cry1Ac.

Binding assays. Immediately before the binding assay, the buffer of the BBMV suspension was replaced with PBS, pH 7.4, containing 0.1% BSA, by microcentrifuge centrifugation. The reaction volume was 0.1 ml, and all samples were duplicated. Other optimal assay conditions were as follows: 0.7 nM ^{125}I-Cry1Ab, 4 μg of BBMV, and 120 min of incubation, for *T. pityocampa* first- and early second-instar larvae; 1.9 nM ^{125}I-Cry1Ab, 12 μg of BBMV, and 60 min of incubation, for *T. pityocampa* last-instar larvae; 0.8 nM ^{125}I-Cry1Ab and 60 min of incubation for *L. monacha* first- and early second-instar larvae; and ^{125}I-labelled toxin (8.7 nM Cry1Aa or 1.3 nM Cry1Ac) and either 60 min (Cry1Aa experiments) or 120 min (Cry1Ac experiments) of incubation for *L. monacha* last-instar larvae. After incubation, the reaction mixtures were filtered through Whatman GF/F glass-fiber filters in a Millipore filtration manifold 1225 Unit (Millipore Corp., Bedford, Mass.). Filters were washed with 5 ml of ice-cold PBS, pH 7.4, containing 0.1% (wt/vol) BSA, and their radioactivity was measured in a 1282 Compugamma CS counter (LKB).

For homologous competition experiments, the reaction mixture contained the corresponding amount of BBMV and labelled toxin and increasing amounts of unlabelled toxin. Binding data were analyzed with the LIGAND program (24), which estimates the binding constants (equilibrium dissociation constant [K_d] and binding site concentration [R_t]). A Student t test was used to determine whether the mean values of the calculated binding constants were significantly different.

TABLE 1. Toxicity of Cry1Aa, Cry1Ab, and Cry1Ac toxins to first-instar *T. pityocampa* larvae[a]

Toxin	Slope ± SE	LC_{50}, pg/μl (90% fiducial limits)
Cry1Aa	1.86 ± 0.36	956 (586–1,552)
Cry1Ab	3.55 ± 1.11	895 (694–1,687)
Cry1Ac	2.68 ± 0.55	379 (279–543)[b]

[a] Slopes and LC_{50} values were determined by the POLO-PC program for dose-mortality curves, LeOra Software.
[b] 95% fiducial limits.

RESULTS

***T. pityocampa* toxicity assays.** Toxicity values for Cry1Aa, Cry1Ab, and Cry1Ac toxins to first-instar *T. pityocampa* larvae are presented in Table 1. Cry1Ac was most toxic, while Cry1Aa and Cry1Ab were equally toxic (95% fiducial limits overlap).

During processionary moth larval development, 1,000 pg of Cry1Ab per μl (concentration around the Cry1Ab LC_{50} value obtained in first-instar larvae) produced only 10% mortality when applied to either second- or third-instar larvae. However, 400 pg of Cry1Ac per μl (also corresponding to the Cry1Ac LC_{50} value obtained in first-instar larvae) caused 35% mortality in second-instar larvae and 10% mortality in third-instar larvae. Hence, the susceptibility of second-instar larvae to Cry1Ab and Cry1Ac toxins is markedly different. A concentration of 10,000 pg of Cry1Ab per μl (a toxin concentration 10-fold higher than the respective LC_{50} values in first-instar larvae, which produced 100% lethality in first-instar larvae) resulted in 10% mortality in second-instar larvae. Even a 4,000-pg/μl Cry1Ac concentration (corresponding to a 100% lethal concentration in first-instar larvae) caused between 40 and 60% mortality in second-instar larvae. These findings stress that second-instar larvae display a different susceptibility to Cry1Ab and Cry1Ac, Cry1Ab being less toxic than Cry1Ac.

Binding to *T. pityocampa* midgut brush border membrane. To analyze the cellular basis for the different toxin susceptibilities of different stages of *T. pityocampa* larval development, tissue sections from early first- and third-instar *T. pityocampa* larvae were prepared and binding of Cry1Ab and Cry1Ac toxins was analyzed. Cry1Ab and Cry1Ac both were able to specifically bind to the midgut brush border membrane of first- and third-instar larvae (Fig. 1), confirming the existence of specific binding sites for these *B. thuringiensis* toxins (28). Controls without toxin or primary antibody were also included (data not shown).

To investigate the biochemical basis of the observed developmental variations in Cry1Ab toxicity, binding saturation experiments with ^{125}I-labelled Cry1Ab were performed. Cry1Ab binding to BBMVs from first- and last-instar *T. pityocampa* larvae followed sigmoidal kinetics (Fig. 2). Binding of Cry1Ab to BBMVs varied with larval development, maximum binding being shown to be between 80 and 150 μg of BBMV/ml, depending on the developmental stage analyzed (Fig. 2). The sigmoidal shape of the curve observed with last-instar larvae (Fig. 2B) indicated a decrease in binding affinity when compared with the hyperbolic curve obtained in first-instar larvae (Fig. 2A).

Quantitative estimates of homologous binding competition experiments are shown in Fig. 3. Equilibrium dissociation constants and binding site concentrations for Cry1Ab binding to first- and last-instar *T. pityocampa* larvae were as follows. For

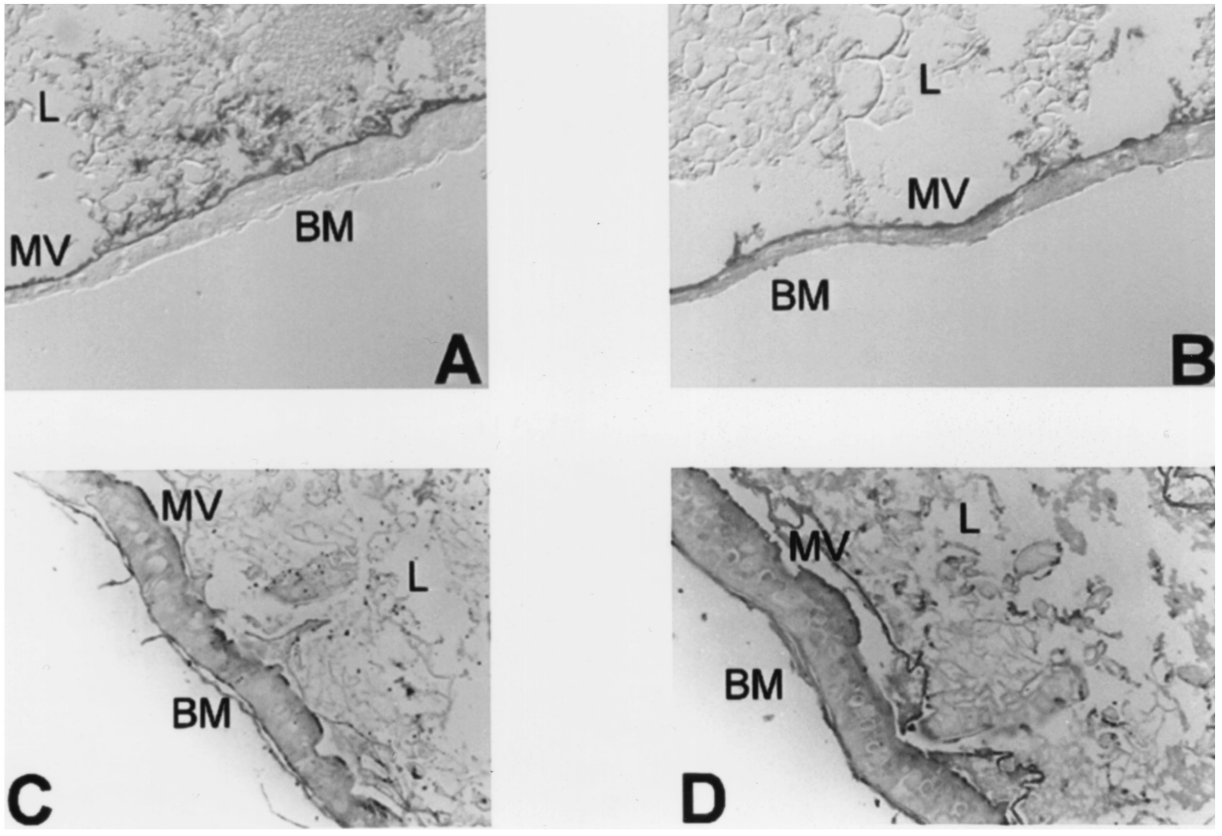

FIG. 1. Binding of Cry1Ab and Cry1Ac to brush border membranes of first- and third-instar *T. pityocampa* larva midguts. (A) Cry1Ab binding to first-instar larva tissue sections; (B) Cry1Ac binding to first-instar larva tissue sections; (C) Cry1Ab binding to third-instar larva tissue sections; and (D) Cry1Ac binding to third-instar larva tissue sections. BM, basal membrane; MV, microvilli; L, lumen.

first- and second-instar larvae, the K_{d1} (± standard deviation [SD]) was 0.29 ± 0.17 nM; the K_{d2} was 2.55 ± 1.62 nM. The R_{t1} was 0.95 ± 0.41 pmol/mg; the R_{t2} was 2.75 ± 1.74 pmol/mg. For last-instar larvae, the K_d (± SD) was 3.42 ± 0.66 nM, and the R_t was 1.29 ± 0.06 pmol/mg. In first-instar larvae, Cry1Ab exhibits one additional high-affinity site which is absent from last-instar larvae. The loss of the Cry1Ab high-affinity binding site during development is in accordance with the decrease in affinity observed in the saturation curve obtained in last-instar larvae (Fig. 2B) compared with that of first-instar larvae (Fig. 2A). In first-instar larvae, saturation is reached at around 80 μg of BBMV protein/ml, whereas, in last-instar larvae, only 25% of maximum binding was detected for that BBMV protein concentration.

The lack of Cry1Ab binding to the high-affinity site in last-instar larvae is consistent with the decrease in toxicity and binding to tissue sections of this toxin during processionary moth development.

Larval growth inhibition assays on *L. monacha*. Preliminary bioassays with *L. monacha* showed a low toxicity of Cry1A toxins compared with that for other insects, such as *T. pityocampa*. Due to this fact, we performed larval growth inhibition experiments with Cry1Aa, Cry1Ab, and Cry1Ac toxins instead of dose-mortality curves. The effects of three sublethal concen-

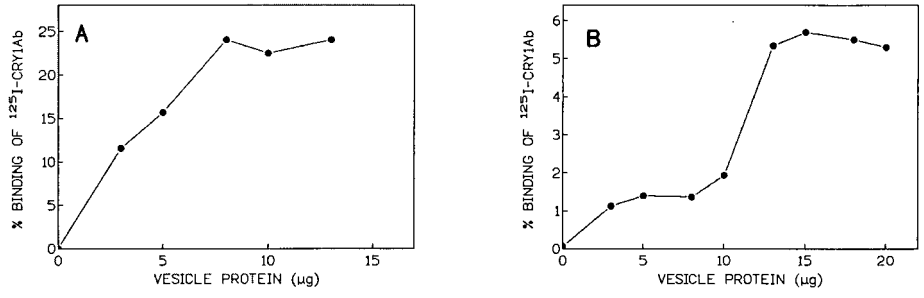

FIG. 2. Specific binding of ^{125}I-labelled Cry1Ab toxin as a function of BBMV concentration of first-instar *T. pityocampa* larvae (A) or last-instar *T. pityocampa* larvae (B). For each point, nonspecific binding (percentage of the total ^{125}I-Cry1Ab counts that were bound to the BBMVs in the presence of a 500-fold excess of unlabelled Cry1Ab) was subtracted from total binding (percentage of the total ^{125}I-Cry1Ab counts that were bound to the BBMVs).

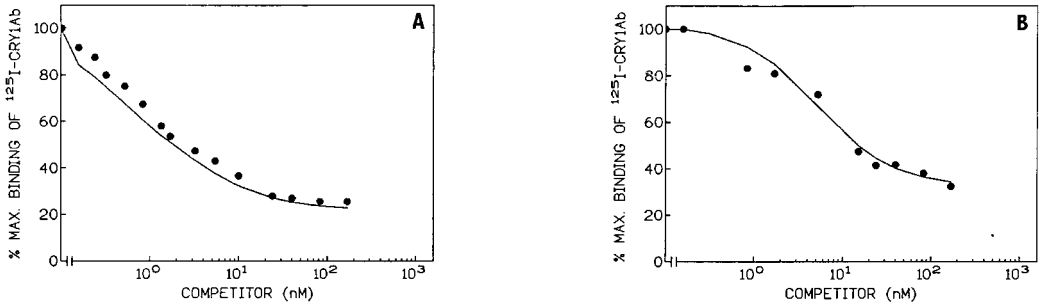

FIG. 3. Binding of ^{125}I-labelled Cry1Ab toxin as a function of increasing concentration of nonlabelled Cry1Ab to BBMVs of first-instar *T. pityocampa* larvae (A) or last-instar *T. pityocampa* larvae (B). For each panel, data points correspond to one of at least three independent competition experiments performed.

trations of each toxin are shown in Fig. 4. In these experiments, as well as in controls with nontreated larvae, mortality never exceeded 5% and 87% of nontreated larva controls survived until the third instar. Results showed around 100% inhibition of molting from second to third instar when a 200-ng/cm^2 concentration of Cry1A-type toxins was used. Fifty percent of the larvae did not molt when exposed to a 2-ng/cm^2 concentration of Cry1Aa, while no significant differences with controls were found when the same Cry1Ab or Cry1Ac concentrations were applied. A 10-fold-higher concentration of Cry1Ac (20 ng/cm^2) was needed to give rise to 50% molting inhibition, while for the same Cry1Ab concentration no significant differences with controls were observed.

In conclusion, larval growth was significantly affected by toxin concentration and the order of relative efficiency in molting inhibition was as follows: Cry1Aa > Cry1Ac > Cry1Ab.

Binding to *L. monacha* midgut BBMVs. In order to determine whether the observed *L. monacha* larval growth inhibition can be related to the occurrence of specific binding of toxins to BBMV, binding experiments with radiolabelled toxins were carried out. Saturable binding of ^{125}I-Cry1Aa and ^{125}I-Cry1Ac to BBMVs prepared from last-instar *L. monacha* larvae was evident (Fig. 5A and B). However, no binding of ^{125}I-Cry1Ab was detected at this developmental stage (Fig. 5C), which contrasted with the observed effect of Cry1Ab on molting inhibition. BBMVs from first- and second-instar larvae were then prepared, and binding saturation experiments with ^{125}I-Cry1Ab were performed. As in the case of *T. pityocampa* with Cry1Ab, results showed saturable binding of Cry1Ab to BBMVs of early stages of *L. monacha* (Fig. 5D).

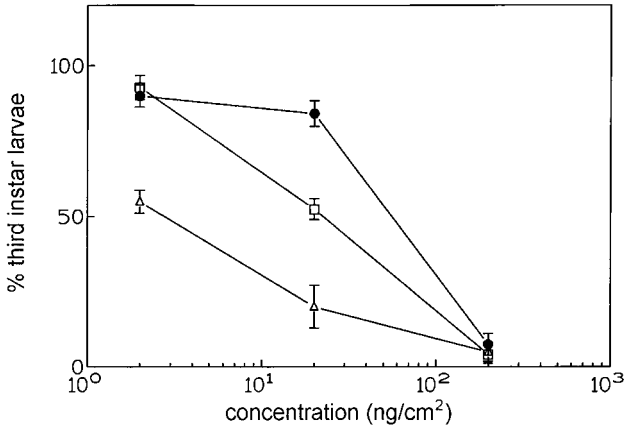

FIG. 4. Percentage of *L. monacha* second-instar larvae that molted to the third instar when sublethal concentrations of Cry1Aa, Cry1Ab, and Cry1Ac were applied on an artificial diet. Percentages were calculated over the total surviving larvae after treatment. In all experiments (including the control, nontreated larvae), mortality never exceeded 5%. The control, nontreated larvae, showed 87% molting to the third instar. Symbols: △, Cry1Aa; ●, Cry1Ab; and □, Cry1Ac.

DISCUSSION

In this paper, the insecticidal activity of Cry1A-type *B. thuringiensis* toxins on *T. pityocampa*, and thus their suitability for processionary moth control, was demonstrated.

In addition, we demonstrated that the toxicity of Cry1Ab and Cry1Ac during larval development decreased with increasing age. A loss of toxicity in later instars is well documented and has been found for several insect pests in relation to *B. thuringiensis* (1–3, 9, 18, 19, 22, 29, 31, 35, 38), showing that the toxicity was mainly determined by the developmental stage of the larvae, i.e., it was significantly higher for earlier stages when compared with later stages. The biochemical basis of the reduced susceptibility among instars has so far been investigated only in *Spodoptera* spp. (19, 22), in which a variation of toxin receptor density during larval development has been proposed to account for the decrease in the capacity of Cry1C and Cry1D toxins to induce permeability changes on BBMVs in late instars (22).

In addition, experimental data indicated a loss or affinity reduction of one of the two Cry1Ab binding sites during development, which may account for the reduced Cry1Ab susceptibility with increasing *T. pityocampa* larval age. To our knowledge, *T. pityocampa* is the first insect pest in which changes in *B. thuringiensis* toxin binding sites during development have been reported. The occurrence of these changes may have a significant influence on the final efficacy of the formulations used against this insect, because larvae in the field are not synchronized and different instars coexist in infested areas. Thus, according to our results, formulations having a higher content of toxins whose activity is not readily lost during development would be more appropriate than those enriched in other toxins whose toxic effect decreases during larval development, as in the case of Cry1Ab in this insect.

On the other hand, in *L. monacha*, growth inhibition assays with sublethal doses of Cry1Aa, Cry1Ab, and Cry1Ac have shown that all three toxins were able to delay development from second to third instar. We have also demonstrated that Cry1Aa and Cry1Ac toxins specifically bound to last-instar *L. monacha* larva BBMVs whereas Cry1Ab did not, although Cry1Ab saturable binding to the brush border membrane of first-second instar *L. monacha* larvae was indeed observed. Therefore, the decrease in Cry1Ab toxicity during development due to the loss of binding sites in later instars could be a

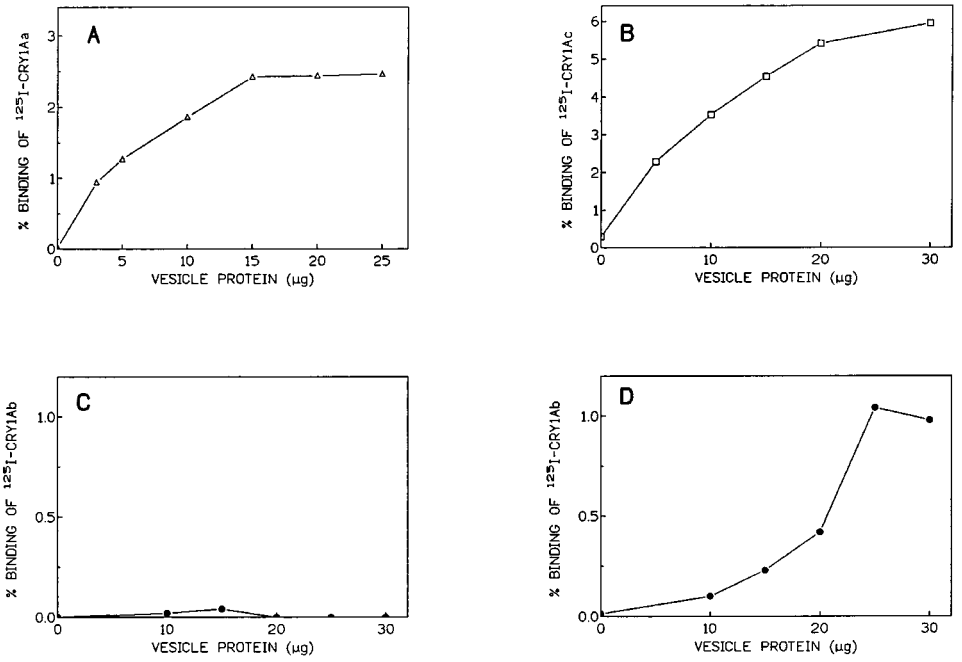

FIG. 5. (A) Specific binding of ^{125}I-Cry1Aa to increasing concentrations of BBMVs from last-instar *L. monacha* larvae. (B) Specific binding of ^{125}I-Cry1Ac to increasing concentrations of BBMVs from last-instar *L. monacha* larvae. (C) Specific binding of ^{125}I-Cry1Ab to increasing concentrations of BBMVs from last-instar *L. monacha* larvae. (D) Specific binding of ^{125}I-Cry1Ab to increasing concentrations of BBMVs from first- and second-instar *L. monacha* larvae. In all cases, the reaction volume was 0.1 ml and for each point nonspecific binding was subtracted from total binding (see the legend to Fig. 2).

more general phenomenon, present not only in *T. pityocampa* but also in other insects.

A similar rationale could be applied to *Lymantria dispar*. In this insect, an inverse correlation has been reported between toxicity and binding properties of Cry1Ab and Cry1Ac to BBMV (39), Cry1Ab being significantly more toxic than Cry1Ac albeit binding with lower affinity. Surprisingly, when we reexamined the experiments of Wolfersberger (39) we realized that toxicity assays were performed on first-instar larvae whereas homologous competition experiments were done with BBMVs from last-instar larvae. Again, a decrease in or loss of Cry1Ab affinity for its binding sites during development could explain this disagreement between toxicity and binding data.

The finding of the variation of Cry1Ab binding sites during development can be explained by a differential expression pattern of the binding molecule in various larval stages. Alternatively, specific posttranslation modifications during development could also be responsible for the decrease in or loss of Cry1Ab binding. The overall effect of either of these changes could have important implications in the characterization of the toxin binding molecules as well as in the design and application of *B. thuringiensis* formulations appropriate for each insect pest.

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