ELSEVIER

Biochimica et Biophysica Acta 1562 (2002) 63–69

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Pore formation activity of Cry1Ab toxin from *Bacillus thuringiensis* in an improved membrane vesicle preparation from *Manduca sexta* midgut cell microvilli

Alejandra Bravo*, Raúl Miranda, Isabel Gómez, Mario Soberón

Departamento de Microbiología, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apartado Postal 510-3, Cuernavaca, Morelos 62250, Mexico

Received 30 October 2001; received in revised form 15 January 2002; accepted 15 January 2002

Abstract

The pore formation activity of Cry1Ab toxin is analyzed in an improved membrane preparation from apical microvilli structures of *Manduca sexta* midgut epithelium cells (MEC). A novel methodology is described to isolate MEC and brush border membrane vesicles (BBMV) from purified microvilli structures. The specific enrichment of apical membrane enzyme markers aminopeptidase (APN) and alkaline phosphatase (APh) were 35- and 22-fold, respectively, as compared to the whole midgut cell homogenate. Ligand-blot and Western-blot experiments showed that Cry1A specific receptors were also enriched. The pore formation activity of Cry1Ab toxin was fourfold higher in the microvilli membrane fraction that showed low intrinsic K^+ channels and higher APN and APh activities than in the basal-lateral membrane fraction harboring high intrinsic K^+ channels. These data suggest that basal-lateral membrane was separated from apical membrane. This procedure should allow more precise studies of the interaction of Cry toxins with their target membranes, avoiding unspecific interaction with other cellular membranes, as well as the study of the pore formation activity induced by Cry toxins in the absence of endogenous channels from *M. sexta* midgut cells.

Keywords: *Bacillus thuringiensis*; Pore formation; Microvilli; Brush border membrane vesicle

1. Introduction

Studies with isolated brush border membranes represent a powerful tool in the analysis of cellular mechanism involved in the transmembrane and transepithelial transport of various solutes, and in the analysis of the interaction of bacterial toxins with their receptors and their pore formation activity. However, data obtained with isolated membrane fractions need careful interpretation since cross-contamination and vesicle heterogeneity can lead to conclusions not related with intact ephitelia.

The midgut of lepidopteran larvae is composed of two major cell types, the columnar cells with an apical brush border microvilli and the goblet cells containing a large cavity which is joined to the apical surface by a complex interdigitated valve [1]. The epithelial cells are joined by septate junctions and gap junctions [2]. The gap junctions provide the electrical and chemical coupling between the globet and columnar cells [3]. The function of the lepidopteran gut is dominated by the vigorous electrogenic transport of K^+ from the blood side to the lumen side [4]. K^+ channels are located in the basal membrane [3–5]. The K^+ proton pump (V-ATPase) and the H^+/K^+ exchanger are located on the apical membrane of the goblet cell [6,7]. Their activity leads to the electrogenic flux of K^+ from the goblet cell cytoplasm to the goblet cell cavity and hence to the gut lumen.

Bacillus thuringiensis (Bt) is an aerobic, spore-forming bacteria that produces crystalline inclusions during the sporulation phase, which are toxic to larvae of several insects orders as well as to other invertebrates [8]. The crystals are composed of proteins known as Cry toxins [9]. It is widely accepted that the primary action of Bt toxins is to lyse midgut ephitelial cells in the target insect [10]. The ingested Cry proteins are dissolved in the alkaline environment of the larval midgut and are cleaved by midgut proteases. The active toxin fragment binds to specific membrane receptors on the apical brush border of the midgut epithelium columnar cells [11,12]. After binding,

* Corresponding author. Tel.: +52-73-291635; fax: +52-73-172388.
E-mail address: bravo@ibt.unam.mx (A. Bravo).

0005-2736/02/$ - see front matter
PII: S0005-2736(02)00360-7

the toxin or part of it, inserts into the cell membrane [13], leading to the formation of lytic pores [14–16], which disrupt midgut ion gradients and the transepithelial potential difference. This disruption is accompanied by an inflow of water that leads to cell swelling and eventual lysis, resulting in paralysis of the midgut and subsequent larval death.

The pore formation activity of purified Cry toxins has been studied in brush border epithelial membranes containing specific receptors [14,17–19]. The method most frequently used to isolate brush border membrane vesicles (BBMV) from lepidopteran insects involves differential Mg^{2+} precipitation introduced by Wolfersberger et al. [20]. However, most BBMV preparations have intrinsic K^+ channels, indicating that the basal-lateral membrane is a cross-contaminant frequently found in the BBMV preparation [14,21,22]. The intrinsic channel activity interferes with the analysis of the pore formation activity of Cry toxins. In this work, we described an improved methodology to purify the brush border membranes from the Cry1A susceptible insect *Manduca sexta*. This novel methodology increases 35- and 22-fold the purification ratio of enzyme markers associated with brush border membranes, aminopeptidase (APN) and alkaline phosphatase (APh), as compared to the whole midgut cell homogenate. Also, the presence of intrinsic K^+ channels is highly reduced. This novel BBMV preparation will be highly useful in the analysis of the Cry toxin ion induced permeability.

2. Materials and methods

2.1. Cry1Ab δ-endotoxin purification

Cry1Ab crystals were produced in the acrystalliferous *Bt* strain 4Q7*cry*$^-$ transformed with pHT315 plasmid [23] harboring the *cry*1Ab gene (pHT315-1Ab) (GenBank accession no. M13898). The transformant strain was grown for 3 days at 29 °C in nutrient broth sporulation medium [24] supplemented with 10 μg/ml erythromycin. After complete sporulation, crystals were purified by sucrose gradients as reported [25], solubilized at 37 °C for 2 h in extraction buffer (50 mM Na_2CO_3 pH 10.5, 0.2% β-mercaptoethanol) and digested with *M. sexta* midgut juice in a 1:10 protease/protoxin mass ratio for 2 h at 30 °C. PMSF was added to a final 1 mM concentration to stop proteolysis. Samples were centrifuged at 16,000 × *g* for 10 min and the toxin-containing supernatant was harvested.

2.2. Isolation of BBMV

M. sexta eggs were kindly supplied by Dr. Jorge Ibarra from CINVESTAV, Irapuato. Larvae were reared on an artificial diet as described [26]. Thirty *M. sexta* larvae in fourth instar were chilled for 10 min on ice and midgut tissue was dissected. The midgut tissue was cleaned from traquea, Malpiglian tubules, peritrophic membrane and midgut content. Then, midguts were cut longitudinally, rinsed with a physiological saline solution (PBS) and incubated for 30–60 min, at room temperature with slow agitation in 100-ml PBS supplemented with Ca^{2+} chelants (EDTA 5 mM and EGTA 5 mM) and protease inhibitors (1 mM PMSF and 100 μg/ml leupeptin). Midgut epithelium cells (MEC) were dissociated using mild agitation of the tissue with forceps, cells were collected by centrifugation 5 min at 120 × *g* (1000 rpm in a bench centrifuge Eppendorf 5804R), washed three times with PBS and suspended in 4-ml cold PBS. Isolated MEC were homogenized by gentle mechanical disruption (2–4 min in vortex maximal velocity) to detach the complete microvilli structure from the disrupted cells. Disrupted cells were loaded in two 10-ml linear density gradient of 0% to 30% Percoll (Sigma Chemical, St. Louis, MO) in PBS, centrifuged 10 min at 2500 × *g* (4500 rpm using a swing rotor in a bench centrifuge Eppendorf 5804R) at 4 °C to purify the microvilli-containing fraction. The two gradients were then fractionated in 10 tubes (1.2 ml each). The microvilli structure sediments in the 25–30% Percoll fractions. The microvilli fraction was suspended in 150 mM KCl, 10 mM HEPES–HCl pH 8, and washed three times by centrifugation for 10 min at 3000 × *g* (5000 rpm Eppendorf 5804R) at 4 °C. Finally, this fraction was sonicated for six periods of 30 s each at 25 °C (Branson 1200 sonic bath, Danbury, CT) in the same solution.

Alternatively, the microvilli fractions were washed and suspended in 300 mM mannitol, 2 mM DTT, 5 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 150 μg/ml pepstatin, 100 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, 10 mM HEPES–HCl, pH 8.0. BBMV were prepared from this fraction by homogenization in a glass and teflon homogenizer, using nine strokes at 3000 rpm. An equal volume of cold 24 mM $MgCl_2$ solution was added to the microvilli homogenate and incubated 15 min at 4 °C. Then it was centrifuged at 2500 × *g* (4500 rpm in a Beckman JA20 rotor) for 15 min at 4 °C. The supernatant was centrifuged at 30,000 × *g* (16,000 rpm Beckman JA20 rotor) for 30 min at 4 °C; the resulting pellet contains the BBMV. The BBMV preparation was dialyzed overnight against 400 volumes of 150 mM KCl, 10 mM HEPES–HCl pH 8, and sonicated as described above in the same solution.

2.3. Enzyme assays

Cytochrome *c* oxidase activity was determined to ensure that microvilli fraction and BBMV were not contaminated with mitochondrial membranes. It was assayed using reduced cytochrome *c* acid modified from horse heart as substrate in a double beam SIM Aminco DW-2000 [27]. APN activity was assayed using L-leucine-*p*-nitroanilide (LpNA) as substrate [28] and APh using *p*-nitrophenyl phosphate as substrate [29]. Protein content was measured by the D_C protein-dye method (Bio-Rad Richmond, CA) using bovine serum albumin as standard (New England Bio-

Labs, Beverly, MA). The initial rate at 405 nm (Ultrospec II spectrophotometer; Pharmacia LKB) was used to calculate specific enzymatic activity of both enzymes. The absorption coefficient of *p*-nitroanilide used was 9.9×10^{-3} mol l^{-1} [30]. One unit of specific APN activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of LpNA min^{-1} mg protein $^{-1}$ at 25 °C. One unit of specific alkaline phosphatease (APh) activity was defined as the amount of enzyme producing 1 μmol of nitrophenol min^{-1} mg protein $^{-1}$ at 25 °C. A standard curve of 4-nitrophenol in 0.5 mM $MgCl_2$, 100 mM Tris pH 9.5 was performed.

2.4. Fluorescence measurements

Membrane potential was monitored with the fluorescent positively charged dye, 3,3′ -dipropylthiodicarbocyanine (Dis-C_3-(5), Molecular Probes, Eugene, OR; 1.5 μM final, 1 mM stock in DMSO). Fluorescence was recorded at 620/670 nm excitation/emission wavelength pair using an Aminco SLM spectroflorometer, as in Lorence et al. [14]. Hyperpolarization causes dye internalization into the BBMV and fluorescence decrease; depolarization causes the opposite effect. Dye calibration and determination of resting membrane potential were performed in the presence of valinomycin (2 μM) by successive additions of KCl to BBMV (10 μg) suspended in 150 mM *N*-methyl-D-glucamine chloride (MeGluCl), 10 mM HEPES–HCl pH 8 buffer (1.8 ml). All determinations were made at 25 °C with constant stirring. Time zero (t_o) was considered when BBMV were added, and toxin (50 nM) addition was made after 2 min. After the first hyperpolarization produced by the toxin, successive additions of KCl (4, 8, 12, 16, 32 and 64 mM, final concentration) to the BBMV were done. After each KCl addition a new membrane potential is established, and a depolarization is produced. The slope (m) of the curve ΔF (%) vs. K^+ equilibrium potential (E_K+) (mV) or vs. external K^+ concentration was determined. E_K+ was calculated with the Nernst equation. Changes in fluorescence determinations were done four times.

2.5. Detection of APN by Western blot

Sandwich Western blots were used to detect APN. Briefly, homogenate, microvilli and BBMV samples were separated by 9% SDS-PAGE and blotted onto nitrocellulose. The blots were incubated with anti-APN [31] kindly supplied by Dr. Michael Adang (Athens GA) (1:10,000 in PBS), and anti-rabbit horseradish peroxidase (1:3000 in PBS). Blots were developed by enhanced chemiluminescence (ECL, Amersham) as described by manufacturers.

2.6. Toxin overlay assay

Protein blot analysis of microvilli and BBMV preparations was done as previously described [32]. Ten micrograms of BBMV protein was separated by 9% SDS-PAGE and electrotransferred to nitrocellulose membranes. After renaturation and blocking, blots were incubated for 2 h with 10 nM of biotinylated Cry1Ab toxin in washing buffer (0.05% Tween 20 in PBS) at room temperature. Unbound toxin was removed by washing three times for 10 min in washing buffer and bound toxin was identified by incubating the blots in TBS containing streptavidin–peroxidase conjugate (1:5000 in PBS) for 1 h. The excess of streptavidin was removed by washing in three changes of washing buffer and the membrane-bound complex was visualized using luminol as above.

3. Results

3.1. Purification of M. sexta BBMV from isolated microvilli

Pore formation activity of Cry toxins has been assayed in BBMV usually prepared from whole larvae midgut tissues [20]. BBMV are prepared by using a differential precipitation with Mg^{2+} that theoretically separates apical and basal-lateral membranes from epithelial cells according to their difference in surface charge density. In a concentration between 15 and 30 mM the divalent cations aggregate basal-lateral membranes as well as mitochondria, lysosomes, and endoplasmic reticulum. Apical membrane fragments do not aggregate [33]. However, preparation of BBMV using whole midgut homogenates as starting material, only increases the apical membrane enzymes markers APh and APN by 6- and 7-fold, respectively [20], and the membrane vesicles contains endogenous K^+ channels [14,22]. Intrinsic K^+ channels interfere with the characterization of Cry1A-induced K^+ permeability. Therefore, we decided to prepare BBMV from microvilli brush border structures that were purified from MEC. Midguts were isolated from 4th instar *M. sexta* larvae and incubated with Ca^{2+} chelants (EDTA and EGTA) to induce epithelium cell detachment from basal lamina (Fig. 1A). MEC were homogenized by gently mechanical disruption to detach the microvilli from the cells. Microvilli structures were then purified by a density 0–30% Percoll gradient as described in Section 2 (Fig. 1B). Microscopic observation of the different Percoll gradient fractions revealed that fractions 1–3 contain intracellular materials and microsomal membranes, fractions 4–8 contains disrupted midgut cell fragments while the higher density fractions 9 and 10 were enriched in microvilli structures (Fig. 1B).

3.2. BBMV marker enzyme enrichment

APN and APh specific activities were determined in the different fractions obtained from the Percoll density gradient. Fig. 2 shows that both APN and APh were enriched in the fractions where microvilli were isolated. The enrichment of these two enzymes in relation to the initial homogenate was only three and four times for APh and APN, respec-

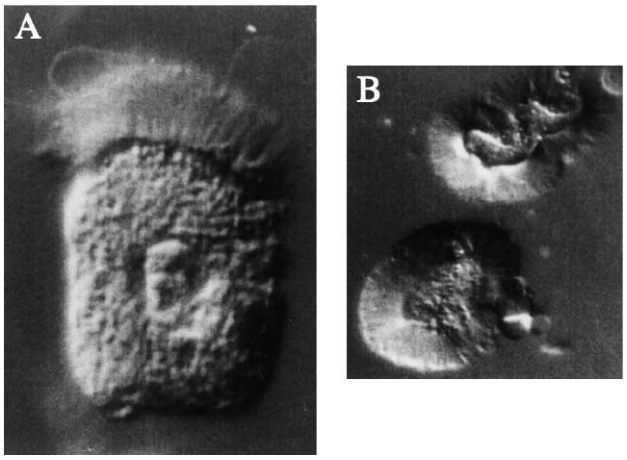

Fig. 1. Light micrographs under Normaski differential interference contrast of isolated *M. sexta* MEC (A) and microvilli structures purified from disrupted cells (B), magnification 100×.

tively (Table 1). However, it is clear from Fig. 2 that the higher activity of both enzymes was found in the fractions of the gradient of higher Percoll concentration that contain the microvilli membranes.

In order to improve the quality of the membrane preparation, membrane vesicles were prepared from isolated microvilli structures using differential Mg^{2+} precipitation. APN and APh enzymatic activities were determined after membrane vesicles isolation. Table 1 shows that BBMV isolated from microvilli have APh and APN enrichments

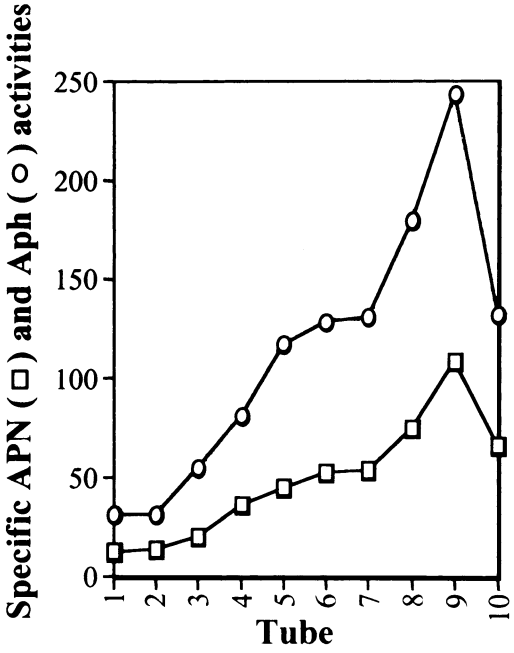

Fig. 2. Microvilli enzyme markers (APN and APh) are enriched in the fractions containing isolated microvilli structures. Analysis of APN (□) and APh (○) specific activities (μmol min^{-1} mg $protein^{-1}$) of the fractions obtained after Percoll gradient centrifugation of disrupted midgut cells.

Table 1
Distribution of APN and APh enzymatic activities in subcellular fractions of *M. sexta* larval midgut

Enzyme	Specific activity (μmol min^{-1} mg $protein^{-1}$)			
	Homogenate	Microvilli	BBMV[a]	BBMV/ homogenate
APh	52 ± 4	249 ± 10	1153 ± 36	22
APN	27 ± 12	109 ± 25	925 ± 52	34

[a] BBMV were purified from isolated microvilli structures as described in Section 2.

of 22- and 34-fold, respectively, in relation to the initial homogenate.

3.3. GPI-anchored APN enrichment and association of Cry1Ab toxin with membrane proteins.

The presence of the Cry1A toxin receptor, a GPI-anchored APN, was analyzed by Western-blot using an antibody raised against a protein fragment of purified *M. sexta* APN [31]. The APN was greatly enriched in the BBMV obtained from isolated microvilli structures (Fig. 3A). Fig. 3B shows a Cry1Ab toxin overlay assay of the three different membrane preparations, initial midgut cells homogenate, microvilli structures and the BBMV isolated from microvilli structures. Toxin overlay assays identify proteins in the membrane that bind Cry1Ab toxin. Fig. 3B shows that besides the 120-kDa APN, the 210-kDa cadherin-like receptor (Bt-R_1) was also present in the microvilli membrane preparations.

3.4. Pore formation activity of Cry1Ab toxin

Pore formation activity of Cry1Ab toxin was assayed in the different fractions obtained after Percoll gradient. The membrane vesicles were loaded with KCl by sonication and pore formation activity was assayed by monitoring changes

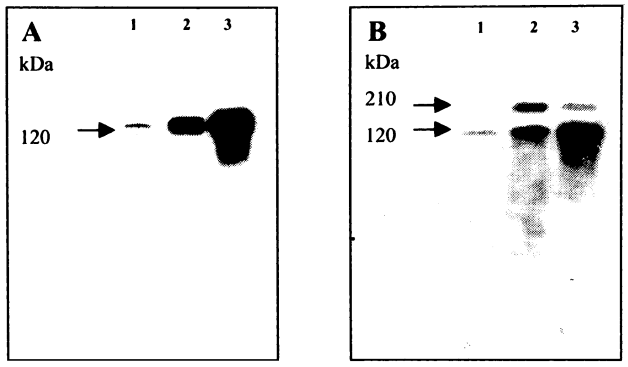

Fig. 3. Cry1A toxin receptors are present in the purified microvilli membranes. Detection of GPI-anchored APN by Western-blot analysis with anti-APN polyclonal antibody (A); toxin overlay assay of biotinylated Cry1Ab to the different membrane samples (B). Homogenate proteins (lines 1), microvilli fraction 9 from the Percoll gradient (lines 2) and BBMV isolated from microvilli structures by the differential Mg^{2+} precipitation method (lines 3).

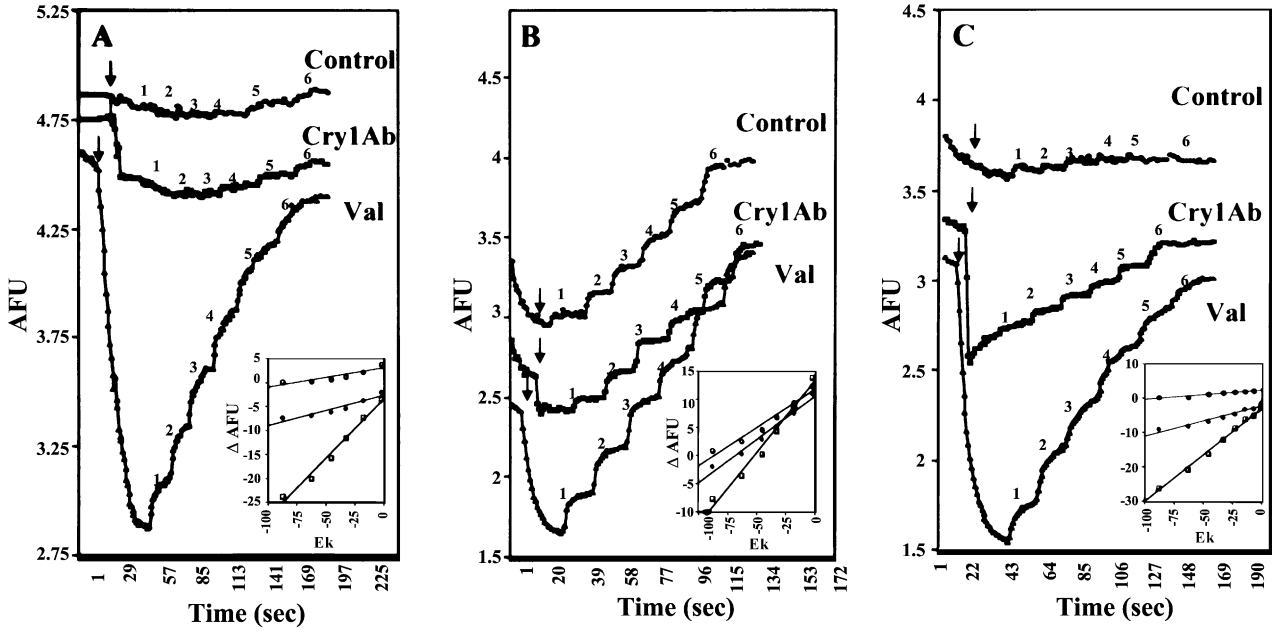

Fig. 4. Analysis of K^+ permeability across membrane vesicles of three different fractions of the Percoll gradient used for purification of microvilli membranes. Membrane vesicles were prepared from fraction 2 that contains intracellular membranes (A), from fraction 5 that contains basal and lateral membranes (B) and from fraction 9 that harbors the purified microvilli membranes (C). Changes in distribution of the fluorescent dye Dis-C_3-(5) sensitive to changes in membrane potential were recorded as described in Section 2. The arrow on top of the traces corresponds to the time of valinomycin, buffer or toxin addition. An upward deflection indicates a membrane potential depolarization; the opposite effect indicates a hyperpolarization. AFU = arbitrary fluorescence units. Final K^+ concentrations (mM) were: 1 = 4, 2 = 8, 3 = 12, 4 = 16, 5 = 32 and 6 = 64. Inserts show the plot of changes in AFU (%) vs. K^+ potential (Ek) (mV) induced by intrinsic K^+ channels (○), valinomycin (□), or by Cry1Ab-activated toxin (◇).

in the fluorescence of a membrane potential-sensitive fluorescent dye Dis-C_3(5) as described in Section 2. Fig. 4 shows the fluorescent traces obtained from three different fractions of the gradient, fraction 2 that contains intracellular membranes (Fig. 4A), fraction 5 that contains principally basolateral membranes (Fig. 4B) and fraction 9 that harbors the purified microvilli membranes (Fig. 4C). All fractions produced similar K^+ permeability response when assayed with the K^+ ionophore valinomycin (Fig. 4). In contrast, the intrinsic K^+ channels were only present in fraction 5 correlating with the presence of basal-lateral membranes (Fig. 4B). Finally, addition of 50 nM of the activated toxin Cry1Ab produced a fast hyperpolarization and an increase in the response of the dye to KCl additions principally in fraction 9 containing the microvilli membranes (Fig. 4C). The plot of the changes in fluorescence vs. K^+ equilibrium potential is shown in the small insert within each panel. The slope of these curves correlates with K^+ permeability across the membrane. Fig. 5 summarizes this information by analyzing the pore formation activity induced by valinomycin, by the intrinsic channels and by the Cry1Ab toxin in all fractions of the Percoll gradient. The Cry1Ab K^+ permeability was calculated by subtracting intrinsic K^+ permeability ($m_{tox} - m_{int}$). This figure shows the percentage of the maximal K^+ permeability obtained from the slope of changes in fluorescence vs. K^+ equilibrium potential. As stated above, all fractions presented similar pore formation when an excess of valinomycin is added; these data indicate

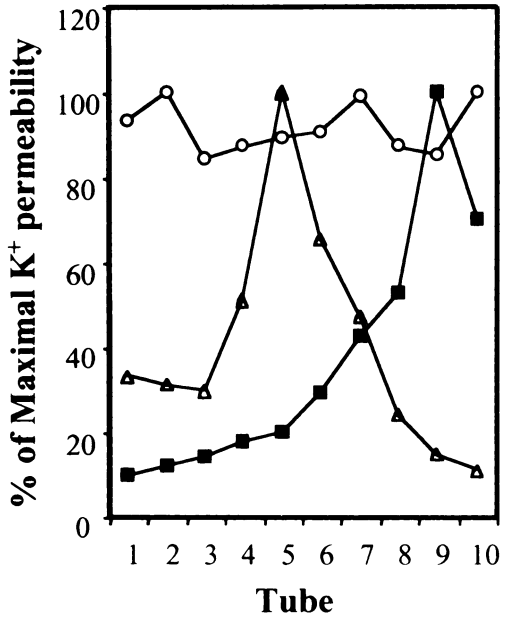

Fig. 5. Percentage of maximal K^+ permeability across membrane vesicle fractions obtained after Percoll gradient centrifugation, produced by intrinsic K^+ channels (△), by valinomycin (○), or by Cry1Ab-activated toxin (■). Changes in distribution of the fluorescent dye (Dis-C_3-(5)) were recorded as described in Section 2. Percentage values were obtained from the slope (m) of the curve ΔF (%) vs. E_{K^+} as in inserts of Fig. 4.

that all fractions contained sealed vesicles that presented a K^+ gradient. The fractions of medium density (fractions 4 to 7) showed a higher intrinsic K^+ permeability, while the Cry1Ab-dependent pore-formation activity was highly increased in the fractions containing the microvilli structures (fractions 9 and 10).

4. Discussion

The pore-forming Cry toxins exert their effect through a multistep process including protoxin proteolytic activation, receptor binding, membrane insertion, oligomerization and finally pore formation. Most of these events have been studied in vitro by the isolation of midgut BBMV, which are the target of the Cry toxins [13]. The purification of BBMV is of key importance to avoid misleading interpretations of the interaction of the toxin with other contaminating cell membranes. For the isolation of BBMV, whole midguts homogenates are differentially precipitated with divalent cations that preferentially precipitate basal-lateral and internal cellular membranes [33]. Using this procedure, enzymatic markers of brush border membranes as APN and APh are enriched sevenfold when compared to the midgut homogenate [20]. In this work we developed a method to purify BBMV from isolated microvilli structures rather than from whole midgut homogenate. The procedure described here showed an enrichment of APN and APh of 34- and 22-fold, respectively, compared with the whole midgut cell homogenate (Table 1), showing that a higher purity preparation of BBMV can be obtained. Ligand-blot and Western-blot experiments demonstrated that Cry1A receptors were enriched in BBMV obtained by this procedure (Fig. 3). In contrast with the 120-kDa APN receptor, the 210 kDa Bt-R_1 receptor was not increased in the final BBMV preparation. This result could suggest a different distribution of these receptors in the midgut cell membrane. The specific enrichment of microvilli by gentle disruption of midgut cells following a density gradient separation (Fig. 1) was illustrated by the separation of membranes harboring low intrinsic K^+ channels, and high Cry1Ab-dependent K^+ permeability (Fig. 4C) of membranes that harbor high intrinsic K^+ channels (Fig. 4B). K^+ ionic channels have been shown to be present in basal-lateral membranes [3,5]. In contrast, purification of BBMV from different larvae (*M. sexta*, *Lymantria dispar* and *Spodoptera frugiperda*) by the differential Mg^{2+} precipitation method had led to the isolation of membranes contaminated with intrinsic channels that were slightly cationic and ranged from 16 to 850 pS [14,21,22].

The procedure described here for BBMV preparation should allow more precise studies of the interaction of Cry toxins with their target membranes avoiding unspecific interaction with other cellular membranes, as well as the study of the pore formation activity induced by Cry toxins in the absence of other intrinsic channels.

Acknowledgements

We thank Oswaldo López, Jorge Sánchez, Lizbeth Cabrera and Javier Luévano for technical assistance. This research was supported in part by DGAPA/UNAM IN216300 CONACyT G36505-N and CONACyT 25248-N.

References

[1] D.F. Moffett, A. Koch, The insect goblet cell: a problem in functional architecture, NIPS 7 (1992) 19–23.
[2] N.J. Lane, H.L. Skaer, Intercellular junctions in insect tissues, Adv. Insect Physiol. 15 (1980) 35–213.
[3] J.A.T. Dow, J.M. Peaccock, Microelectrode evidence for the electrical isolation of Goblet cell cavities in *Manduca sexta* middle midgut, J. Exp. Biol. 143 (1989) 101–114.
[4] D.F. Moffett, A. Koch, Electrophysiology of K^+ transport by midgut epithelium of lepidopteran insect larvae I. The transbasal electrochemical gradient, J. Exp. Biol. 135 (1988) 25–38.
[5] J.A.T. Dow, Insect midgut function, Adv. Insect Physiol. 19 (1986) 187–238.
[6] W.R. Harvey, S. Nedergaard, Sodium independent active transport of potassium in the isolated midgut of the cecropia silkworm, Proc. Natl. Acad. Sci. U.S.A. 51 (1964) 757–765.
[7] D.F. Moffett, A. Koch, Electrophysiology of K^+ transport by midgut epithelium of lepidopteran insect larvae II. The transapical electrochemical gradients, J. Exp. Biol. 135 (1988) 39–49.
[8] R. de Maagd, A. Bravo, N. Crickmore, How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world, TIG 17 (2001) 193–199.
[9] N. Crickmore, D.R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, D.H. Dean, Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins, Microbiol. Mol. Biol. Rev. 62 (1998) 807–813.
[10] B.H. Knowles, Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins, Adv. Insect Physiol. 24 (1994) 274–308.
[11] A. Bravo, S. Jansens, M. Peferoen, Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects, J. Invertebr. Pathol. 60 (1992) 237–246.
[12] C. Hofmann, H. Vanderbruggen, H. Höfte, J. Van Rie, S. Jansens, H. Van Mellaert, Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high affinity binding sites in the brush border membrane of target insect midgut, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 7844–7848.
[13] E. Schnepf, N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler, D.H. Dean, *Bacillus thuringiensis* and its pesticidal crystal proteins, Microbiol. Mol. Biol. Rev. 62 (1998) 775–806.
[14] A. Lorence, A. Darszon, C. Díaz, A. Liévano, R. Quintero, A. Bravo, δ-endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers, FEBS Lett. 360 (1995) 217–222.
[15] J.L. Schwartz, L. Garneau, L. Masson, R. Brousseau, E. Rousseaeu, Lepidopteran-specific crystal toxins from *Bacillus thuringiensis* form cation- and anion-selective channels in planar lipid bilayers, J. Membr. Biol. 132 (1993) 53–62.
[16] P.V. Pietrantonio, S.S. Gill, *Bacillus thuringiensis* δ-endotoxins: action on the insect midgut, in: M.J. Lehane, P.F. Billingsley (Eds.), Biology of the Insect Midgut, Chapman & Hall, London, 1996, pp. 345–372.
[17] J. Carroll, D.J. Ellar, An analysis of *Bacillus thuringiensis* delta-endotoxin action on insect midgut membrane permeability using a light scattering assay, Eur. J. Biochem. 214 (1993) 771–778.
[18] J. Carroll, D.J. Ellar, Analysis of the large aqueous pores produced by a *Bacillus thuringiensis* protein insecticide in *Manduca sexta*

midgut brush border membrane vesicles, Eur. J. Biochem. 245 (1997) 797–804.
[19] K. Hendrickx, A. de Loof, H. Van Mellaert, Effects of *Bacillus thuringiensis* delta-endotoxin on the permeability of brush border membrane vesicles from tobacco hornworm (*Manduca sexta*) midgut, Comp. Biochem. Physiol. 95C (1990) 241–245.
[20] M. Wolfersberger, P. Lüthy, A. Maurer, F. Parenti, V. Sacchi, B. Giordana, G.M. Hanozet, Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*), Comp. Biochem. Physiol. 86A (1987) 301–308.
[21] F.G. Martin, M.G. Wolfersberger, *Bacillus thuringiensis* delta-endotoxin and larval *Manduca sexta* midgut brush border membrane vesicles act synergistically to cause very large increases in the conductance of planar lipid bilayers, J. Exp. Biol. 198 (1995) 91–96.
[22] O. Peyronnet, V. Vachon, J.L. Schwartz, R. Laprade, Ion channel activity from the midgut brush-border membrane of gypsy moth (*Lymantria dispar*) larvae, J. Exp. Biol. 12 (2000) 1835–1844.
[23] O. Arantes, D. Lereclus, Construction of cloning vectors for *Bacillus thuringiensis*, Gene 108 (1991) 115–119.
[24] D. Lereclus, H. Agaisse, M. Gominet, J. Chaufaux, Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis spo*OA mutant, Bio/Technology 13 (1995) 67–71.
[25] W.E. Thomas, D.J. Ellar, *Bacillus thuringiensis* var. israelensis crystal δ-endotoxin: effect in insect and mammalian cells in vitro, J. Cell Sci. 60 (1983) 181–197.
[26] R.A. Bell, F.G. Joachim, Techniques for rearing laboratory colonies of tobacco budworms and pink bollworms, Ann. Entomol. Soc. Am. 69 (1976) 365–373.
[27] J. García-Soto, M. González-Martínez, L. de la Torre, A. Darszon, Sea urchin sperm head plasma membranes: characteristics and egg jelly induced Ca^{2+} and Na^{+} uptake, Biochim. Biophys. Acta 944 (1988) 1–12.
[28] A. Lorence, A. Darszon, A. Bravo, The pore formation activity of *Bacillus thuringiensis* Cry1Ac toxin on *Trichoplusia ni* membranes depends on the presence of aminopeptidase N, FEBS Lett. 414 (1997) 303–307.
[29] E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, p. 597.
[30] J.C.M. Hafkenscheid, Enzymes 3; peptidases, proteinases and their inhibitors, in: J. Bergemayer (Ed.), Methods of Enzymatic Analysis, vol. V, Verlag Chemie, Weinheim, 1984, pp. 2–34.
[31] K. Luo, J.R. McLachlin, M.R. Brown, M.J. Adang, Expression of glycosylphosphatidylinositol-linked *Manduca sexta* aminopeptidase N in insect cells, Protein Expression Purif. 17 (1999) 113–122.
[32] E. Aranda, J. Sánchez, M. Perferoen, L. Güereca, A. Bravo, Interaction of *Bacillus thuringiensis* crystal protein with the migdut epithelial cells of *Spodoptera frugiperda* (Lepidoptera: Noctuidae), J. Invertebr. Pathol. 68 (1996) 203–212.
[33] R. Kinne, H. Murer, L. Kinne-Saffrano, M. Thees, G. Sachs, Sugar transport by renal plasma membrane vesicles: characterization of the systems in the brush border microvilli and basal-lateral plasma membranes, J. Membr. Biol. 22 (1975) 375–395.