ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 2, pp. 202-208. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © N. V. Bulushova, D. P. Zhuzhikov, L. I. Lyutikova, N. E. Kirillova, I. A. Zalunin, G. G. Chestukhina, 2011, published in Biokhimiya, 2011, Vol. 76, No. 2, pp. 245-252.

Toxin-Binding Proteins Isolated from Yellow Mealworm *Tenebrio molitor* and Wax Moth *Galleria mellonella*

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Received September 22, 2010
Revision received October 19, 2010

Abstract—A 67-kDa protein that can specifically bind the activated Cry9A endotoxin under ligand-blotting conditions was purified from midgut epithelium apical membranes of wax moth *Galleria mellonella* by affinity chromatography. N-Terminal amino acid sequencing enabled identification of this protein as aminopeptidase N. In similar experiments, 66- and 58-kDa proteins specific to endotoxin Cry3A were isolated from the midgut epithelium apical membranes of *Tenebrio molitor* larvae. Mass spectrometry showed close similarity of the 58-kDa protein to the *Tenebrio molitor* α-amylase.

DOI: 10.1134/S0006297911020064

Key words: δ-endotoxin, *Bacillus thuringiensis*, *Galleria mellonella*, *Tenebrio molitor*, toxin-binding protein

δ-Endotoxins (Cry proteins) of *Bacillus thuringiensis* comprise a large family of proteins that selectively kill larvae of many insects belonging to the orders Lepidoptera, Diptera, Coleoptera, as well as nematodes [1]. More than 100 Cry proteins possessing similar spatial structure are now known, but they differ in their primary structures and spectra of insecticide activity [2-4]. For instance, Cry11A is effective against mosquito larvae, Cry3A against beetle grubs, while members of Cry1 and Cry9 classes are toxic for many caterpillars [5]. Some Cry proteins, in particular the Cry9 endotoxins, have a molecular mass ranging within 130-145 kDa and are protoxins undergoing proteolytic activation to form activated toxins with molecular masses of 65-70 kDa [1]. Other Cry proteins, such as endotoxins Cry3A and Cry11A, have molecular masses of 65-70 kDa and correspond to the activated first type toxins in their spatial structure and function [2, 3]. The activated toxins bind with specific proteins (so-called toxin-binding proteins or receptors) exposed on the apical membrane (brush border) of the insect midgut epithelial cells and oligomerize to form transmembrane pores or ion channels [1]. Identification of receptors in organisms of sensitive insects is necessary for revealing the mechanisms underlying specificity of these proteins, adaptation of pathogenic microorganisms to their host organism, and appearance of resistant insect forms, which should provide development of next-generation bioinsecticides. The following classes of membrane-associated proteins have been identified in insect midgut epithelium as receptors of Cry toxins: cadherin-like proteins, aminopeptidases N, alkaline phosphatases [1], α-amylases [6], ADAM metalloproteinase [7], V-ATPase subunits A and B [8, 9], and some others.

In this work we used affinity chromatography to isolate specific Cry-binding proteins from the apical membranes of midgut epithelium of yellow mealworm *Tenebrio molitor* and wax moth *Galleria mellonella* larvae (receptors of Cry3A and Cry9A endotoxins, respectively) [10, 11].

MATERIALS AND METHODS

Isolation of δ-endotoxins. The following *B. thuringiensis* strains were used in this work: the strain belonging to ssp. *tenebrionis* was provided from Sandoz Crop Protection Corporation (USA), and strains B-1757 (ssp. *galleriae*) and B-2395 (ssp. *israelensis*) from the National Collection of Industrial Microorganisms (Russian

Abbreviations: AM (PM), anterior (posterior) midgut; BBMV, brush border membrane vesicles.

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acronym VKPM). The microorganisms were grown in liquid medium containing 1% trypcasin (Oltoanyagtermelo es Kutato Intezet, Hungary), 0.2% yeast extract (Serva, Germany), and 0.6% glucose at 28°C until complete lysis of sporangia [12]. Crystals of endotoxins were separated from other components of cell autolysate in a biphasic xylene–water system [12].

Cry3A endotoxin was prepared by dissolution of crystals from ssp. *tenebrionis* in 0.1 M sodium carbonate buffer, pH 11 (buffer A), for 1 h at 28°C under continuous agitation. The solution was dialyzed overnight against 0.1 M sodium carbonate buffer, pH 9.5. A 49-kDa Cry3A fragment ($Cry3A_{49}$) having the toxicity of the native endotoxin against mealworm [13] but significantly more soluble at pH 5-9 [14] was prepared by limited proteolysis of the initial protein with chymotrypsin (Worthington Biochemical, USA) by the method of Carroll and associates [14] with slight modifications [13].

The activated Cry9A endotoxin ($Cry9A_{65}$) was prepared by treatment of crystals from strain B-1757 with 0.1 M sodium carbonate buffer, pH 10.3, containing 10 mM dithiothreitol (DTT; Serva), 5 mM EDTA (Sigma-Aldrich, USA), and 1 mM diisopropylfluorophosphate (Fluka, USA) for 1 h at 20°C under agitation. The mixture was centrifuged for 20 min at 15,000*g* in a Beckman J2-21 centrifuge (GMI, USA). The supernatant was subjected to limited proteolysis with trypsin (Serva) and anion-exchange chromatography on a HiTrap Q column (GE Healthcare, Sweden) as described earlier [15]. The $Cry9A_{65}$ protein was concentrated on a Mono S cation-exchange column (GE Healthcare) equilibrated with 50 mM Tris-HCl buffer, pH 8.8. The protein was eluted with 0.2 M NaCl in the same buffer.

The Cry11A endotoxin was purified from the insecticide crystals of strain B-2395 by the selective dissolution method [16].

Protein was determined by the Bradford method [17].

Biotinylation of proteins. Before biotinylation, toxins and products of their limited proteolysis were dialyzed against 0.1 M carbonate buffer, pH 9.5, containing 0.15 M NaCl. Biotinylation was carried out using biotin-N-hydroxysuccinimide ester (Amersham Biosciences, United Kingdom), according to the previously described method [18].

Isolation of brush border membrane vesicles (BBMV) from *T. molitor* and *G. mellonella* larvae. *Tenebrio molitor* and *G. mellonella* larvae were grown as described earlier [19, 20]. Third-stage larvae were used for experiments. The larvae were dissected and the part of the digestive tract corresponding to the midgut or its anterior (anterior midgut, AM) or posterior (posterior midgut, PM) half were excised. The excised specimens were washed with 17 mM Tris-HCl buffer, pH 7.5, containing 0.3 M mannitol (Sigma-Aldrich) and 5 mM EGTA (Sigma-Aldrich) (buffer B), transferred to a fresh aliquot of the same buffer, and stored at –80°C before use.

The midgut specimens were thawed and homogenized in buffer B containing 1% (v/v) protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich). BBMV was isolated by the previously described method including precipitation of unwanted membranes with magnesium ions by differential centrifugation [21, 22]. The purity degree of apical membranes was estimated by comparing specific activities of the marker enzyme, leucyl aminopeptidase, in the initial homogenate and in the purified specimen (leucine-*p*-nitroanilide was used as substrate) [22].

Synthesis of affinity sorbents. Affinity sorbents ($Cry3A_{49}$)- and ($Cry9A_{65}$)-aminohexyl-agarose were synthesized using ω-aminohexyl-agarose (Sigma-Aldrich) activated with glutaraldehyde (Merck, Germany) as described earlier [23]. The prepared ($Cry3A_{49}$)-aminohexyl-agarose contained 2.0 mg (41 nmol) of protein per ml sorbent, and the ($Cry9A_{65}$)-aminohexyl-agarose contained 1.5 mg (23 nmol) of protein per ml of sorbent.

Determination of protein composition of *T. molitor* membrane specimens and their extracts prepared by treatment with nonionic surfactant. To determine the protein composition of vesicles, a sample (4 μl) of *T. molitor* AM or PM BBMV was dissolved in sample buffer containing 0.05 M Tris-HCl, pH 8.0, 1% SDS (Sigma), 0.01 M DTT, and 10% glycerol (Serva) and analyzed by SDS-PAGE. To prepare extract of membrane-associated proteins, 4 μl of the above vesicle sample was mixed with 0.1 ml 1% *n*-octyl glycoside (Boehringer Mannheim, Germany) in 0.05 M sodium carbonate buffer, pH 9.5, containing protease inhibitor cocktail and incubated for 30 min in an ice bath. Following centrifugation for 15 min at 17,000*g* and 4°C on an Eppendorf 5417R centrifuge (Eppendorf, Germany), the protein composition of the extracts was determined by SDS-PAGE.

Electrophoresis. Samples were analyzed by SDS-PAGE in 10% gel by the method of Laemmli [24]. Samples (10 μl) were mixed with an equal volume of 2× sample buffer and incubated for 5 min at 100°C. A multicolor protein ladder (11-170 kDa) (Fermentas, Lithuania) was used as the molecular mass standard.

Identification of Cry3A-binding proteins in *T. molitor* BBMV. A sample (4 μl) of *T. molitor* AM or PM BBMV was subjected to SDS-PAGE followed by electrotransfer onto a nitrocellulose membrane (Sigma-Aldrich) in a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad, USA) for 5 h at a current of 65 mA. Following a typical incubation in PBS containing 0.3% Tween 20 and 1% ovalbumin (Reanal, Hungary), membranes were incubated with one of the following biotinylated proteins: Cry3A, $Cry3A_{49}$, or BSA (Sigma) (protein concentration 8 or 4 μg/ml) for 1 h under agitation and treated with the biotin–streptavidin system as described earlier [18]. The nitrocellulose membranes were stained for protein with 0.1% Ponceau S in 1% acetic acid.

Affinity chromatography of *T. molitor* membrane-associated protein extract. An aliquot (0.4 ml) of *T. moli-*

tor PM BBMV was mixed with 2 ml of 0.05 M Tris-HCl buffer, pH 8.6, containing 0.05 M EDTA, 1% protease inhibitor cocktail, and 1% *n*-octyl glycoside (buffer C) and incubated as described above. Thus prepared extract was applied onto a column filled with 2 ml of ($Cry3A_{49}$)-aminohexyl-agarose and equilibrated with 0.05 M Tris-HCl buffer, pH 8.2, containing 10 mM of EDTA and 0.2% *n*-octyl glycoside (buffer D) at flow rate 0.1 ml/min. Chromatography was carried out according to a slightly modified protocol described earlier [18]. The column was sequentially washed with buffer D and 1 M NaCl solution in buffer D. Toxin-binding proteins were eluted with buffer A. Collected fractions were pooled, dialyzed against buffer D, and stored at −80°C.

Affinity chromatography of *G. mellonella* membrane-associated protein extract. An aliquot (1.4 ml) of *G. mellonella* BBMV was mixed with 2 ml of buffer C and incubated as described above. Thus prepared extract was applied onto a column filled with 2 ml of ($Cry9A_{65}$)-aminohexyl-agarose equilibrated with buffer D. The column was washed as described above, and toxin-binding protein was eluted with PBS containing 0.2% *n*-octyl glycoside and 0.1 M N-acetylgalactosamine. Fractions were collected and stored at −80°C.

Analysis of affinity chromatography fractions by ligand blotting. The protein fractions eluted from the affinity columns were analyzed by Laemmli's SDS-PAGE followed by electrotransfer onto nitrocellulose membrane as described above. The membranes were incubated with one of the following biotinylated proteins: Cry11A, $Cry9A_{65}$, $Cry3A_{49}$, or BSA (Sigma) (protein concentration 8 or 4 μg/ml) for 1 h under agitation and then treated with the biotin–streptavidin system.

Homologous and heterologous competition. Nitrocellulose filters with sorbed Cry3A- or Cry9A-binding proteins were incubated with biotinylated $Cry3A_{49}$ and $Cry9A_{65}$ (4 μg/ml), respectively, either taken alone or mixed with 20-fold excess of unlabeled protein – either the same one (homologous competition) or (in experiments with Cry3A-binding proteins) its expected competitor $Cry9A_{65}$ or Cry11A (heterologous competition). Subsequent treatment was as described above.

N-Terminal amino acid sequencing. Fractions eluted from the affinity columns were concentrated 20-fold by ultrafiltration through a PM30 membrane (Millipore, USA). Trichloroacetic acid was added to 100 μl of the concentrate up to the final concentration of 7% and centrifuged for 5 min at 8000*g*. The pellets were dissolved in 10 μl of sample buffer and separated by SDS-PAGE followed by electrotransfer onto an Immobilon-P^{SQ} membrane (Millipore). The N-terminal amino acid sequence was determined using an Applied Biosystems 470A automated gas-phase protein sequencer (USA).

Identification of proteins using mass spectrometry. Proteins separated in polyacrylamide gel were hydrolyzed with trypsin, and peptide mass fingerprints were determined by tandem MALDI-TOF spectrometry using a Bruker Ultraflex II MALDI-TOF/TOF spectrometer equipped with a neodymium UV laser (Germany). Fast polarity switching enabled the acquisition of both positive and negative ions online, and the accuracy of monoisotopic mass measurement in reflectron mode was 0.007%.

The NCBI database was searched for all proteins with given accuracy and taking in account possible oxidation of methionine residues by oxygen and modification of cysteine residues by acrylamide using the Mascot search engine (option "peptide mass fingerprint"; www.matrixscience.com).

RESULTS

Isolation and characterization of *T. molitor* BBMV. BBMV specimens were purified from both AM and PM sections of *T. molitor* midgut. In the case of PM, apical membranes were purified 22-fold, while in the case of AM the purification degree was not estimated because of the absence of leucine aminopeptidase activity in the initial homogenate, which is consistent with previously reported data [25]. Another BBMV marker enzyme, alkaline phosphatase, is absent in membranes of both *T. molitor* midgut sections [25]. We could not find the corresponding enzymatic activity either in AM and PM homogenates or in fractions obtained in the course of BBMV isolation. Analysis of total protein composition of vesicles by SDS-PAGE showed a slight difference between AM and PM: both specimens contained proteins with molecular mass ranging for 20 to more than 170 kDa. Similarity of the protein composition can serve as indirect proof of high purity of AM BBMV specimen. The treatment of vesicles with nonionic surfactant, *n*-octyl glycoside, resulted in solubilization of most of the membrane-associated proteins (data not shown).

Identification of Cry3A-binding proteins in *T. molitor* BBMV. Extracts of *T. molitor* larva PM BBMV were subjected to electrophoresis followed by electrotransfer onto a nitrocellulose membrane. Incubation of the resulted replicas with biotinylated toxins Cry3A and $Cry3A_{49}$ showed the presence in both cases of four colored bands with molecular masses of 120, 95, 58, and 30 kDa (Fig. 1). The band corresponding to the 30-kDa protein is exhibited under incubation with the conjugate alone, likely corresponding to unidentified membrane-associated protein containing biotin. In ligand blotting, a relative coloration intensity of bands corresponding to the 95- and 58-kDa proteins dramatically increased as compared with the picture of protein staining, thus making the corresponding components candidates for the role of *T. molitor* BBMV toxin-binding proteins. The band corresponding to a 120-kDa protein is major when the nitrocellulose filters are stained with Ponceau S, so we did not

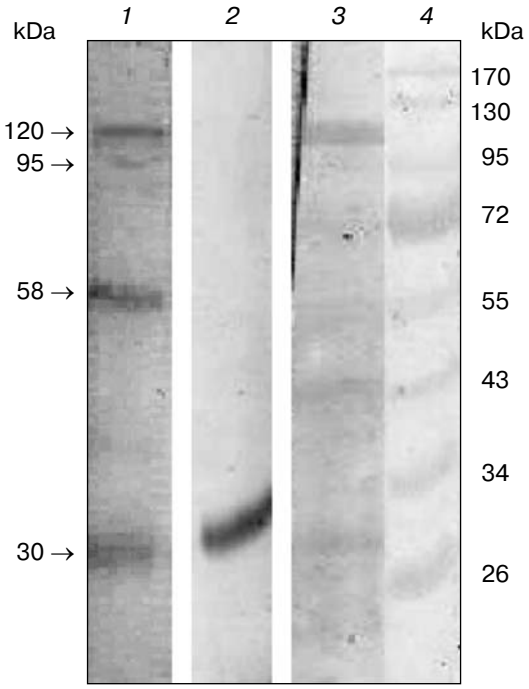

Fig. 1. Toxin-binding proteins from *T. molitor* BBMV visualized using ligand blotting. The *T. molitor* BBMV proteins were subjected to SDS-PAGE followed by electrotransfer. The nitrocellulose replicas were treated as follows: *1*) incubated with biotinylated $Cry3A_{49}$ and stained with the biotin–streptavidin system; *2*) stained similarly, but without preliminary incubation with the toxin; *3*) stained with Ponceau S; *4*) molecular mass standards. Arrows indicate visualized bands with molecular masses of corresponding proteins.

observe its relative color intensification in ligand blotting. Similar experiments with *T. molitor* AM BBMV gave similar results. Subsequent experiments were performed with vesicles from PM.

Affinity chromatography of protein extract from *T. molitor* BBMV. Affinity chromatography of the extract prepared by treatment of *T. molitor* PM BBMV with *n*-octyl glycoside on ($Cry3A_{49}$)-aminohexyl-agarose allowed purification of proteins with molecular masses of 66 and 58 kDa interacting with $Cry3A_{49}$ under ligand-blotting conditions (Fig. 2).

Specificity of Cry3A binding by 66- and 58-kDa proteins from *T. molitor* BBMV. Biotinylated Cry3A binds with the 66- and 58-kDa proteins as well as does its 49-kDa fragment (data not shown). If the corresponding replicas were incubated with the biotinylated toxins that do not possess activity against mealworm larvae, namely anti-mosquito Cry11A and anti-lepidopterous $Cry9A_{65}$ (Fig. 2), as well as with biotinylated BSA, no colored band is observed. The bands corresponding to the 66- and 58-kDa proteins are also absent when nitrocellulose replicas are incubated with biotinylated $Cry3A_{49}$ in the presence of 20-fold excess of the unlabeled toxin (homologous competition). Hence, the binding between the given proteins and $Cry3A_{49}$ does not result from biotinylation of the latter. A 20-fold excess of non-biotinylated Cry11A and $Cry9A_{65}$ (heterologous competition) has no effect on the ability of biotinylated $Cry3A_{49}$ to stain the 66- and 58-kDa bands, also evidence for specificity of this effect. The data are summarized in the table.

Identification of toxin-binding proteins from *T. molitor* BBMV by mass spectrometry. Mass spectrometry revealed a 58-kDa protein eluted from $Cry3A_{49}$-aminohexyl-agarose as *T. molitor* α-amylase (Accession No. P56634; gi|45579533) (*score* value is 115).

Similarly, the 66-kDa band contains Cry3A endotoxin (gi|61221657) (*score* 129). Nevertheless, some analyzed peptides correspond to vacuolar ATPase (V-ATPase) subunit A from red flour beetle *Tribolium castaneum* (Acc. No. XP_976188.1; gi|91081489). In this case, the *score* value was 67.

Isolation of Cry9A-binding protein from *G. mellonella* caterpillar BBMV. BBMV was isolated from the complete midgut of caterpillars; 18-fold degree of purification was achieved. Affinity chromatography of proteins extracted from the purified vesicles with the nonionic surfactant *n*-octyl glycoside on $Cry9A_{65}$-aminohexyl-agarose yielded in a protein with molecular mass of 67 kDa. This protein was hardly eluted with 1 M NaCl, but it effectively eluted with 0.1 M N-acetylgalactosamine. In ligand blotting the 67-kDa protein bound the biotinylated

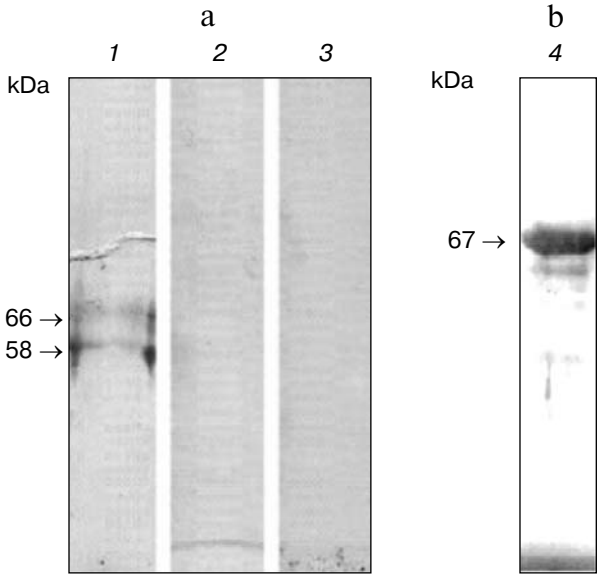

Fig. 2. Binding of proteins eluted from $Cry3A_{49}$- and $Cry9A_{65}$-aminohexyl-agarose with Cry toxins of various specificity. a) Nitrocellulose replicas containing proteins eluted from $Cry3A_{49}$-aminohexyl-agarose were incubated with the following biotinylated toxins: $Cry3A_{49}$ (*1*), Cry11A (*2*), and $Cry9A_{65}$ (*3*). b) Nitrocellulose replica containing a 67-kDa protein eluted from $Cry9A_{65}$-aminohexyl-agarose was incubated with the biotinylated endotoxin $Cry9A_{65}$ (*4*). Arrows indicate visualized bands with corresponding molecular masses.

Specificity of interaction between 66- and 58-kDa proteins from *T. molitor* BBMV and Cry toxins in ligand blotting

Biotinylated toxin (2.5 μg/ml)	Non-biotinylated toxin (50 μg/ml)	Staining of the 66- and 58-kDa protein bands
$Cry3A_{49}$	–	yes
$Cry3A_{49}$	$Cry3A_{49}$	no
$Cry3A_{49}$	$Cry9A_{65}$	yes
$Cry3A_{49}$	Cry11A	yes
$Cry9A_{65}$	–	no
Cry11A	–	no

$Cry9A_{65}$ (Fig. 2), but it did not interact with the biotinylated toxins $Cry3A_{49}$ and Cry11A, which are ineffective against *G. mellonella* caterpillars, or with biotinylated BSA (data not shown), thus suggesting specificity of the binding.

Identification of 67-kDa protein from *G. mellonella* BBMV. N-Terminal sequencing of a 67-kDa protein from *G. mellonella* BBMV revealed the following amino acid sequence: Leu-Asn-Leu-Asn-Gln-Asn-Leu. This shares 71% homology with the peptide Asp-Asn-Leu-Asn-Glu-Asn-Leu, fragment 98-104 of *Plutella xylostella* caterpillar aminopeptidase N4 (Acc. No. AAS75552).

DISCUSSION

Affinity chromatography on resins such as toxin-agaroses seems to be the most effective method for isolation of toxin-binding proteins from insect gut epithelium. Earlier, we used this type of chromatography for isolation of toxin-binding proteins from *Aedes aegypti* [18, 22] and *Anopheles stephensi* [16] mosquito larvae and obtained proteins with molecular masses of 65 and 62 kDa in the first case and 65 and 57 kDa in the second case. These proteins showed alkaline phosphatase activity [16, 26, 27].

In the present work a 67-kDa protein that specifically binds the activated endotoxin Cry9A in ligand blotting was isolated from *G. mellonella* caterpillar BBMV by affinity chromatography on $Cry9A_{65}$-aminohexyl-agarose. A similar method using $Cry3A_{49}$-aminohexyl-agarose applied to *T. molitor* larva PM BBMV yielded 66- and 58-kDa proteins with specific activity to Cry3A endotoxin. The isolated membrane components are similar to the toxin-binding proteins from mosquito larvae in molecular mass, but further studies showed that they belong to different protein classes. Comparison of N-terminal amino acid sequence of the 67-kDa protein from *G. mellonella* BBMV with the protein database of Lepidoptera (taxid: 7088) demonstrated 71% identity with the primary structure of *Plutella xylostella* aminopeptidase N4 between residues 98 and 104 (BLAST software). No other candidates for as orthologs for the studied protein were revealed during the comparison, in particular no identity to Lepidoptera alkaline phosphatases was found. The molecular mass of the isolated protein was substantially lower than those of *P. xylostella* aminopeptidase N4 (106.6 kDa; "Proteins" source in NCBI) and other Lepidoptera aminopeptidases (100-170 kDa) [1, 28] and likely represents a product of limited proteolysis of a presently unknown *G. mellonella* aminopeptidase.

Interestingly, the 67-kDa protein was effectively eluted from an affinity column with N-acetylgalactosamine. This implicates this sugar residue in the interaction between 67-kDa protein and toxin molecules. Similar results were obtained earlier for the binding of Cry1Ac with aminopeptidase and alkaline phosphatase from several caterpillars [29, 30].

The absence of membrane-bound alkaline phosphatase was demonstrated for *T. molitor* larva midgut (the present work, as well as [25, 31]), hence this enzyme cannot serve as a toxin receptor in this insect. Unfortunately, the N-terminal sequencing of 66- and 58-kDa proteins did not yield an unambiguous result. To all appearances, in both cases we deal with a molecular mixture. From the mass-spectroscopy data, the 58-kDa protein was identified as *T. molitor* α-amylase. There are reports that proteins of the α-amylase family serve as receptors for *B. thuringiensis* toxins [6] and for binary toxin of *Bacillus sphaericus* [32]. All these proteins are attached to cellular membrane by GPI-anchor, whereas the presently known *T. molitor* α-amylase is a soluble protein. We have determined that it is devoid of a potential site for GPI-modification using big-PI Predictor-GPI Modification Site Prediction software from ExPASy proteomic tools. However, the 58-kDa protein was isolated from insect BBMV. This suggests that it represents a yet unknown membrane form of *T. molitor* α-amylase. This is indirectly confirmed by the fact that its molecular mass differs from the theoretically calculated molecular mass of soluble enzyme (51.24 kDa). On the other hand, one cannot exclude that soluble *T. molitor* α-amylase can form a complex with membrane proteins of the insect gut epithelium.

Mass spectrometry demonstrated that the material forming the 66-kDa band contains Cry3A endotoxin. Apparently, this is the initial Cry3A that is always present as an admixture in the $Cry3A_{49}$ specimen used for the sorbent synthesis [14]. This protein could partially leak from the sorbent during chromatography. However, the same analysis of the 66-kDa band demonstrates, besides Cry3A, the presence of another protein that most closely

resembles vacuolar ATPase (V-ATPase) subunit A from red flour beetle *Tribolium castaneum*, an insect closely related to *T. molitor* (both species are the members of darkling beetles Tenebrionidae). These facts suggest that the 66-kDa band contains *T. molitor* V-ATPase subunit A (there is no evidence on its structure in databases) that can specifically bind Cry3A. Evidences suggesting that V-ATPase subunit A demonstrates specific affinity to some Cry proteins were obtained earlier when identifying Cry1Ac-binding proteins from *Heliothis virescens* BBMV [8, 33]. V-ATPase subunit B from *Helicoverpa armigera* BBMV has similar properties [9]. V-ATPase is found in BBMV specimens isolated from many insect species [9, 31, 33-37]. This enzyme is virtually unstudied, but it is obviously not identical to the well-studied V-ATPase from goblet cells [38] because of the difference in enzymatic properties [36].

It remains unclear whether or not the indicated V-ATPase subunits can fulfill the role of toxin receptor. They comprise V_1 domain of vacuolar ATPase, which forms a complex with transmembrane V_0 domain of the same enzyme at the inner surface of the plasma membrane [38]. According with a general scheme, Cry proteins bind with a receptor localized on the outer side of apical membrane [1]. However, recent data suggests that some endotoxins can penetrate into epithelial cells via endocytosis [39, 40]. Based on these data, the *H. virescens* V-ATPase subunit A has been called an intracellular Cry1Ac receptor [8].

Recently a cadherin-like protein was shown to operate as a Cry3A receptor in *T. molitor* BBMV [41]. We did not find this protein in fractions eluted from $Cry3A_{49}$-aminohexyl-agarose, probably because of ineffectiveness of affinity chromatography for its isolation. There are many examples of identification of several toxin-binding proteins for a single toxin–insect pair [42-44]. The presence of several receptors in one cell is completely understandable. So, a hypothesis was proposed that the toxins fulfill their function via their successive binding, initially with a cadherin receptor and then with aminopeptidase or other membrane proteins [45, 46]. Besides, the presence of several receptors for a single toxin in insect gut epithelium complicates appearance of resistant insect forms. On the other hand, we suppose that proteins possessing affinity to toxins not always play the role of receptors for these toxins *in vivo*. Some of them can bind Cry proteins without harmful consequences for the cell or even defend it by decreasing the virtual toxin concentration.

Thus, in the course of our studies we isolated toxin-binding proteins from midgut epithelium of *G. mellonella* caterpillars and *T. molitor* grubs. However, identification of a 66-kDa toxin-binding protein from the beetle BBMV requires further confirmation.

This study was supported by RFBR grants Nos. 08-04-00737-a and 09-04-91289-INIS_a.

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