ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 2, pp. 133-139. © Pleiades Publishing, Inc., 2006.
Original Russian Text © M. A. Dronina, L. P. Revina, L. I. Kostina, L. A. Ganushkina, I. A. Zalunin, G. G. Chestukhina, 2006, published in Biokhimiya, 2006, Vol. 71, No. 2, pp. 173-181.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-099, October 23, 2005.

Toxin-Binding Proteins from Midgut Epithelium Membranes of *Anopheles stephensi* Larvae

M. A. Dronina[1], L. P. Revina[1], L. I. Kostina[1], L. A. Ganushkina[2], I. A. Zalunin[1]*, and G. G. Chestukhina[1]

[1]*Scientific Research Institute for Genetics and Selection of Industrial Microorganisms, 1-yi Dorozhnyi Proezd 1, 117545 Moscow, Russia; fax: (7-495) 315-0501; E-mail: zalunin@genetika.ru*
[2]*Sechenov Moscow Medicine Academy, Martsinovsky Institute of Medical Parasitology and Tropical Medicine, ul. Bolshaya Pirogovskaya 2, Korpus 3, 119992 Moscow, Russia*

Received April 13, 2005
Revision received June 17, 2005

Abstract—Proteins of 65 and 57 kD were isolated from the apical membranes of midgut epithelium of *Anopheles stephensi* larvae by affinity chromatography. These proteins can specifically bind endotoxin Cry11A and activate toxin Cry4B (Cry4B-tox) under conditions of ligand blotting, and both Cry proteins compete for this binding. At least in the case of Cry4B-tox, the binding with 65 and 57 kD proteins is reversible. The ability of the products of limited proteolysis of Cry11A and Cry4B to bind the 65 and 57 kD proteins correlates with their toxicity to *A. stephensi* larva. The N-terminal amino acid sequence of the 57 kD protein is unique and absent in the NCBI GenBank. The proteins of 65 and 57 kD share most of the properties studied with *Aedes aegypti* toxin-binding proteins. It is possible that they altogether represent a novel class (or classes) of δ-endotoxin receptors.

DOI: 10.1134/S0006297906020039

Key words: δ-endotoxins, *Bacillus thuringiensis*, toxin-binding proteins, *Anopheles stephensi*

The entomopathogen *Bacillus thuringiensis* (BT) produces entomocide proteins, so-called δ-endotoxins, killing many insect larvae (mainly Lepidoptera, Diptera, and Coleoptera) and also nematodes [1]. The entomocide proteins are synthesized in a bacterial cell during sporulation and form inclusion bodies (crystals) in it [2]. The δ-endotoxins consist of two classes of proteins, Cry- and Cyt-proteins. About one hundred Cry-proteins possessing similar secondary and tertiary structure but differing by spectrum of insect sensitivity are known to date [3, 4]. For example, representatives of Cry1 family are toxic for various Lepidoptera species (lepidocide proteins). The endotoxins Cry4A, Cry4B, Cry11A, and also Cyt1Aa protein kill mosquito larvae (the mosquitocide proteins), whereas endotoxin Cry3A is toxic for some beetle larvae [2, 5].

Most Cry-proteins (e.g., the endotoxins Cry1A, Cry4A, and Cry4B) are protoxins of 130-140 kD; their proteolytic activation involves degradation the C-terminal part of the molecule. The proteolytic cleavage yields the activated toxins of 65-70 kD [6]. Some proteins (e.g., Cry3A and Cry11A) possess initial molecular mass of 65-70 kD, and their spatial structure corresponds to the structure of activated toxins of higher molecular weight Cry-proteins [1]. A molecule of activated toxin contains three domains responsible for various functions [7, 8]. The N-terminal domain is involved in formation of membrane pores or ion channels. Two other domains are responsible for binding to an apical membrane receptor of epithelial cells. Several receptors to lepidocide toxins have been found in the caterpillar gut [1, 9-11]. However, little is known about mosquito larval receptors for the mosquitocide toxins. We previously isolated proteins from *Aedes aegypti* mosquito larvae; they exhibited affinity to the mosquitocide proteins Cry4B and Cry11A [12, 13]. Here we describe isolation of toxin-binding proteins from *Anopheles stephensi* larvae.

MATERIALS AND METHODS

Isolation of δ-endotoxins. The strains of *B. thuringiensis* B-2395 (ssp. *israelensis*) and B-9032 (ssp. *kurstaki*) were from Collection of Industrial Microorganisms, Institute for Genetics and Selection of

* To whom correspondence should be addressed.

Industrial Microorganisms (Moscow); the strain ssp. *tenebrionis* was supplied by Sandoz Crop Protection Corp. (USA). These microorganisms were cultivated at 28°C in a liquid medium containing 1% tripcasin (Human, Hungary), 0.2% yeast extract (Serva, Germany), and 0.6% glucose up to complete sporangia lysis [14]. Endotoxin crystals were separated from other components of autolysates using a biphasic xylene–water system [14].

Endotoxins Cry4B, Cry11A, and Cyt1Aa were isolated from the entomocide crystals of the B-2395 strain. For isolation of endotoxins Cry4B and Cyt1Aa crystals were treated with 0.05 M sodium carbonate buffer, pH 10.5, containing 0.01 M dithiothreitol (DTT) (Serva) (buffer A) at 20°C for 1 h under stirring. The mixture was centrifuged at 15,000*g* for 20 min using a Beckman J2-21 centrifuge (GMI, Inc., USA). The resultant extract was chromatographed using a Mono Q column (Amersham Biosciences, Sweden) equilibrated with 0.05 M sodium carbonate buffer, pH 10.5, containing 1 mM DTT. Proteins were eluted with a linear gradient of NaCl (0-1 M) in the same buffer. The endotoxins Cyt1Aa and Cry4B were eluted at NaCl concentrations of 0.1 and 0.4 M, respectively [15].

For isolation of the Cry11A endotoxin, the pellet, obtained after the procedure described above, was extracted with 0.05 M NaOH at 20°C for 30 min and centrifuged at 15,000*g* for 20 min; the resultant supernatant extract was dialyzed against 0.05 M sodium carbonate buffer, pH 9.5 [16].

The Cry1A endotoxins were obtained by dissolving crystals of B-9032 strain in buffer A for 1 h at 37°C and stirring followed by subsequent dialysis of the resultant solution against 0.05 M Tris-HCl buffer, pH 8.5.

For preparation of the endotoxin Cry3A, ssp. *tenebrionis* crystals were dissolved in 0.1 M sodium carbonate buffer, pH 10.7, at 28°C for 1 h under stirring.

Protein concentration was assayed by the method of Bradford [17].

Limited proteolysis of entomocide proteins. Before the procedure of limited proteolysis, all preparations of the entomocide proteins were dialyzed against 0.05 M Tris-HCl buffer, pH 8.6. Initial preparation of Cry4B endotoxin was treated with chymotrypsin (Serva), trypsin (Serva), or gut juice of *A. aegypti* larvae [12, 13] to yield endotoxin fragments of molecular mass of 64, 50, and 48-49 kD, respectively. The resulting hydrolyzates are defined in the text as Cry4B-tox, Cry4B-tr, and Cry4B-Aa, respectively.

Proteolysis of Cry11A yielding fragments of 33 and 36 kD was carried out at 37°C for 2 h using the enzyme/substrate mass ratio of 1 : 100; this hydrolyzate is further defined as Cry11A-tr.

Hydrolyzate Cry1A-tox was obtained by treating a mixture of Cry1A endotoxins with trypsin (1 : 100). The proteolytic procedure was carried out at 37°C for 12 h.

The table shows characteristics of the proteolytic products.

Protein biotinylation. Before biotinylation, toxins and their proteolytic products were dialyzed against 0.1 M carbonate buffer, pH 9.5, containing 0.15 M NaCl. Biotinylation was carried out using N-succinimide biotin ester (Amersham Biosciences) as described earlier [13].

Preparation of *A. stephensi* larvae gut epithelial brush border membranes (BBM). Larvae were cultivated in water at 25°C using KiteKat dry and vitamins for their nutrition [18]. Midguts were removed from insects reaching the third larval stage under binocular control. Insects were placed onto a microscope glass slide in a drop of 17 mM Tris-HCl buffer, pH 7.5, containing 0.3 M mannitol (Sigma-Aldrich, USA) and 5 mM EGTA (Sigma-Aldrich) (buffer B); the larval body was opened and the part of its gastrointestinal tract corresponding to the midgut was excised. The excised material was washed in buffer B containing 1% (v/v) cocktail of protease inhibitors for mammalian tissues (Sigma-Aldrich), transferred into a fresh volume of the same buffer, and stored at –80°C.

The frozen samples were thawed and homogenized in buffer B containing cocktail of protease inhibitors. BBM were isolated in several stages including magnesium ion membrane sedimentation and differential centrifugation described in details in [12, 19]. Comparison of specific activity of the marker enzyme (leucine aminopeptidase and leucine *p*-nitroanilide as substrate [12]) in the initial homogenate and the resulting preparation revealed 6-fold purification of the apical membranes.

Synthesis of affinity sorbents. The affinity sorbents Cry11A- and Cry4B-Sepharose were synthesized using CNBr-activated Sepharose 4B (Serva) and the method described in [12]. The resulting Cry11A-Sepharose and Cry4B-Sepharose preparations contained 1.6 mg (22.9 nmol) and 1 mg (15.4 nmol) of protein per ml of the sorbent, respectively.

Study of protein composition of membrane protein extracts obtained using various detergents. Portions of BBM preparation (4 μl) were mixed with 0.1 ml of 1% solution of one of the following detergents: NONIDET P40 (Amersham Biosciences), *n*-octylglucoside (Boehringer Mannheim, Germany), and CHAPS (Sigma) in 0.05 M sodium carbonate buffer, pH 9.5, containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and 1 mM leupeptin (Sigma). The mixtures were incubated in an ice bath for 30 min and then centrifuged at 17,000*g* and 4°C for 15 min using a 5417R centrifuge (Eppendorf AG, Germany). Protein composition of the resulting extracts was evaluated using SDS-PAGE.

Affinity chromatography of an extract of *A. stephensi* membrane proteins. A BBM preparation (0.14 ml) was mixed with 2 ml of 0.05 M sodium carbonate buffer, pH 9.5, containing 0.05 M EDTA, 1% cocktail of protease inhibitors, and 1% NONIDET P40; the mixture was incubated as described above. The extract was applied to

a column containing 2 ml of Cry11A- or Cry4B-Sepharose equilibrated with buffer C (0.05 M sodium carbonate buffer, pH 9.5, containing 0.2% NONIDET P40 and 0.01 M EDTA) at the rate 0.1 ml/min. Chromatography was carried out as described earlier [13] with minor changes. Columns were sequentially washed with buffer C and buffer C containing 1 M NaCl. Fractions eluted at 1 M NaCl were kept at –80°C.

Electrophoresis. SDS-PAGE was carried out by the method of Laemmli [20]. Ligand blotting experiments were performed as follows. An aliquot of the analyzed sample (10 μl) was mixed with 10 μl of the sampling buffer (0.05 M Tris-HCl, pH 8.0, 20% (v/v) glycerol, 2% SDS (Sigma), and 0.02 M DTT); the mixture was incubated at 100°C for 5 min. For determination of molecular mass of toxin binding proteins, a fraction eluted from Cry4B-Sepharose was concentrated 20-fold by means of ultrafiltration using PM30 membrane (Millipore, USA). The concentrate (100 μl) was mixed with trichloroacetic acid (final concentration 7%) and centrifuged at 8000*g* for 5 min. The resulting pellet was dissolved in 10 μl of the sampling buffer. Molecular masses were evaluated using a protein marker kit (Sigma) containing bovine serum albumin (BSA) (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), and trypsin inhibitor (20 kD).

Ligand-blotting. In the ligand blotting experiments fractions obtained after affinity chromatography were subjected to gel electrophoresis according to the method of Laemmli followed by subsequent electrotransfer onto nitrocellulose membrane using Trans-Blot SD (Bio-Rad, USA) at 65 mA for 5 h. Nonspecific binding was blocked by incubating with 1% egg albumin in PBS containing 0.3% Tween-20 at 4°C for 12 h. The filters were incubated for 1 h in 0.02 M Tris-HCl, pH 8.6, containing 0.14 M NaCl, 0.3% Tween-20 (buffer D), and one of the biotinylated proteins (8 or 4 μg/ml); after the incubation the filters were washed five times with buffer D (5 ml for each washing) and treated with a streptavidin–peroxidase conjugate (Amersham Biosciences) diluted by 1 : 1000. Protein bands exhibiting biotinylated toxin binding activity were determined using the ECL-Western blotting analysis system (Amersham Biosciences) following the supplier's instructions.

Study of binding of *A. stephensi* proteins of 65 and 57 kD with BT toxins of various specificity and with proteolytic products of the mosquitocide proteins Cry11A and Cry4B. Binding of the 65 and 57 kD proteins with toxins of various specificity was studied by the above described method and using the following biotinylated toxins as putative ligands: Cry11A (4 μg/ml), Cry4B-tox (4 μg/ml), Cyt1Aa (8 μg/ml), Cry1A-tox (8 μg/ml), and Cry3A (8 μg/ml).

Binding of the 65 and 57 kD proteins with products of limited proteolysis of the mosquitocide proteins as putative ligands was studied using the following biotinylated proteins: Cry11A-tr (4 μg total protein per ml), Cry4B-tr (4 μg/ml), and Cry4B-Aa (4 μg total protein per ml).

Experiments on homologous and heterologous competition. The nitrocellulose filters with transferred 65 and 57 kD proteins were incubated with the biotinylated variant of one of the mosquitocide toxins, Cry11A or Cry4B-tox (4 μg/ml), or its mixture with 10-fold excess of the non-biotinylated variant of the same protein (homologous competition) or a putative competitor (Cry4B-tox or Cry11A, respectively) (heterologous competition). Subsequent treatment was carried out as described.

Study of reversibility of mosquitocide protein binding. The nitrocellulose filters containing 65 and 57 kD proteins were incubated with biotinylated Cry4B-tox (4 μg/ml) as described above, washed five times in the incubation buffer, and treated for 1 h with 10-fold excess of the non-biotinylated variant of the same toxin.

Determination of N-terminal amino acid sequence. Five milliliters of the fraction eluted from Cry4B-Sepharose at 1 M NaCl were concentrated 200-fold by ultrafiltration and sedimentation with trichloroacetic acid as described above; the resulting fraction was subjected to SDS-PAGE and electrotransfer onto Immobilon-P^{SQ} membrane (Millipore). The N-terminal amino acid sequences of the protein bands corresponding to 65 and 57 kD proteins were determined using an Applied Biosystems (USA) model 470A gas-phase sequencer.

Search for sequences orthologous to those experimentally found was carried out using the program BLAST Search for short, nearly exact matches and the NCBI GenBank database.

Alkaline phosphatase activity assay. The following samples (2 μl each) were prepared: a) BBM protein extract obtained as described above and diluted 100-fold in 0.05 M sodium carbonate buffer, pH 9.5; b) the fraction eluted from Cry4B-Sepharose at 1 M NaCl and concentrated 10-fold by ultrafiltration; c) 0.02% BSA. These were applied as dots onto a nitrocellulose filter. The latter was carefully washed with 0.05 M sodium carbonate buffer, pH 9.5, and treated with 0.1 M Tris-HCl buffer, pH 9.5, containing 0.1 M NaCl, 0.01 M $MgCl_2$, and substrates of the phosphatase reaction (5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT); Promega, USA). Appearance of lilac color in the corresponding dot indicated the presence of alkaline phosphatase activity.

RESULTS

Affinity chromatography of *A. stephensi* larval proteins from midgut epithelium BBM. Preliminary experiments revealed that extracts obtained by treating BBM preparation of *A. stephensi* larvae with nonionic (NONIDET P40 and *n*-octylglucoside) and zwitterionic (CHAPS) deter-

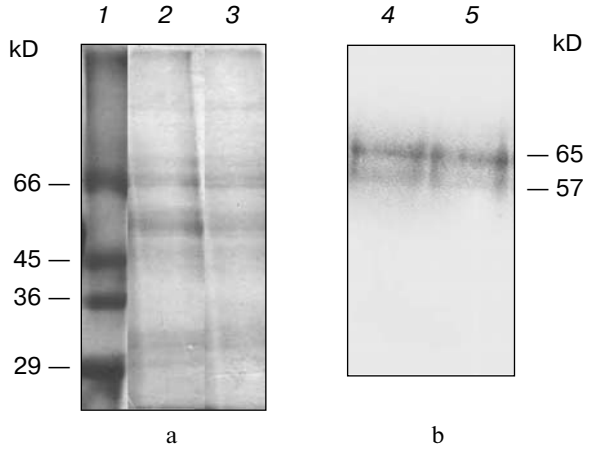

Fig. 1. SDS-PAGE of fractions obtained during affinity chromatography of *A. stephensi* BBM proteins: extract of membrane proteins (lane *2*); Cry4B-Sepharose non-bound proteins (lane *3*); fractions eluted from Cry11A-Sepharose (lane *4*) or Cry4B-Sepharose (lane *5*) at 1 M NaCl. Lane *1* contained molecular weight marker proteins. Gels were stained with Coomassie R-250 (a) or by the method of ligand blotting using biotinylated endotoxin Cry11A as ligand (b).

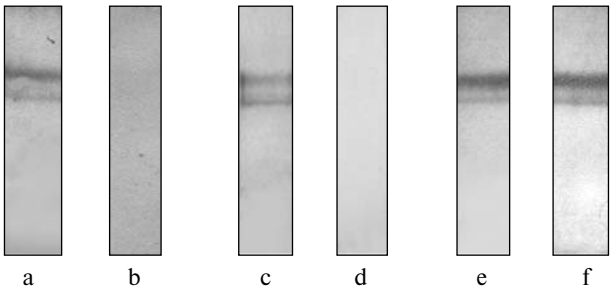

Fig. 2. Binding of 65 and 57 kD proteins of *A. stephensi* BBM with various BT endotoxins and products of their limited proteolysis. The nitrocellulose replicas containing 65 and 57 kD proteins eluted from Cry4B-Sepharose were incubated with the following biotinylated proteins: Cry4B-tox (a); Cry3Aa (b); Cry4B-tr (c); Cry4B-Aa (d); Cry11A (e), Cry11A-tr (f).

gents have similar protein composition and contain proteins ranging from 27 to 100 kD. Figure 1 shows protein composition of the extract obtained using NONIDET P40, which was used in subsequent experiments.

During affinity chromatography on Cry4B- and Cry11A-Sepharose, the major proportion of *A. stephensi* BBM proteins did not bind to the sorbents (Fig. 1). However, in both cases 1 M NaCl eluted proteins of 65 and 57 kD reacting with the biotinylated toxin Cry11A under ligand blotting conditions (Fig. 1).

Interaction of 65 and 57 kD proteins of *A. stephensi* BBM with BT toxins exhibiting various specificity of the entomocide effect. Under ligand blotting conditions, the proteins eluted from Cry11A- and Cry4B-Sepharose at 1 M NaCl react similarly with Cry4B-tox and endotoxin Cry11A (both 4 μg/ml) (Figs. 2 and 3). However, these proteins do not react with biotinylated Cry1A-tox, Cry3A, and Cyt1Aa used in higher concentration (8 μg/ml) (see Fig. 2 for Cry3A endotoxin).

Reversibility of binding of 65 and 57 kD proteins of *A. stephensi* BBM with Cry4B-tox. If the nitrocellulose replicas containing proteins eluted at 1 M NaCl from affinity sorbents (Cry11A- or Cry4B-Sepharose) are initially incubated with biotinylated Cry4B-tox and then with excess of the non-biotinylated variant of the same toxin the detection system used in this study does not exhibit bands corresponding to 65 and 57 kD proteins

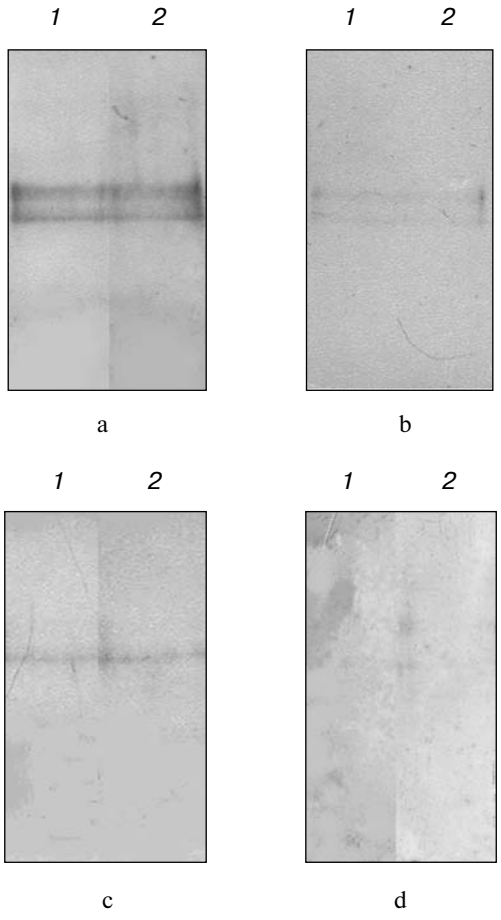

Fig. 3. Homologous and heterologous competition during Cry4B-tox binding to 65 and 57 kD proteins of *A. stephensi* BBM and reversibility of this effect. The nitrocellulose replicas containing 65 and 57 kD proteins eluted from Cry4B-Sepharose (*1*) and Cry11A-Sepharose (*2*) were incubated with the following endotoxins or their mixtures: a) biotinylated Cry4B-tox (4 μg/ml); b) mixture of biotinylated Cry4B-tox (4 μg/ml) and non-biotinylated Cry4B-tox (40 μg/ml); c) mixture of biotinylated Cry4B-tox (4 μg/ml) and non-biotinylated Cry11A (40 μg/ml). In the case of (d), treatment of the nitrocellulose filter with biotinylated Cry4B-tox (4 μg/ml) was followed by its incubation with non-biotinylated variant of this toxin (40 μg/ml).

(Fig. 3). This suggests reversibility of binding of Cry4B-tox with these membrane proteins.

Homologous and heterologous competition during interaction of Cry11A endotoxin and Cry4B-tox with 65- and 57-kD proteins. Treatment of the nitrocellulose replicas containing proteins eluted from the affinity sorbents at 1 M NaCl with a mixture of biotinylated Cry4B-tox and 10-fold excess of its non-biotinylated variant (homologous competition) did not result in subsequent visualization of bands corresponding to 65- and 57-kD proteins (Fig. 3). Similar results were obtained after incubation of the same replica with mixture of biotinylated Cry11A and 10-fold excess of non-biotinylated Cry11A (data not shown).

Under conditions of heterologous competition (replicas were treated with a mixture of biotinylated Cry4B-tox or Cry11A and 10-fold excess of non-biotinylated heterologous proteins, Cry11A or Cry4B-tox, respectively), bands corresponding to 65 and 57 kD proteins were also not observed. Figure 3 illustrates the first of the variants of heterologous competition.

Interaction of 65 and 57 kD proteins with products of limited proteolysis of endotoxins Cry4B and Cry11A. Data of ligand blotting indicate that Cry11A-tr is as active as intact Cry11A in the interaction with 65 and 57 kD proteins. Similar results were obtained for Cry4B-tr. However, Cry4B-Aa did not bind to 65 and 57 kD proteins (Fig. 2).

Determination of N-terminal sequence of *A. stephensi* BBM 57 kD protein. The fraction eluted from Cry4B-Sepharose at 1 M NaCl and concentrated 200-fold was applied to a 10% polyacrylamide gel. The protein bands corresponding to proteins of 65 and 57 kD were cut from the nitrocellulose replica obtained after electrophoresis and subsequent electrotransfer; and the protein material of these bands was analyzed using the automated Edman method.

We failed to read the N-terminal sequence of 65 kD component. In the case of 57 kD component the following sequence was obtained: S-L-H-Q-H-I-F-S-D-L.

Determination of phosphatase activity in the eluate containing 65 and 57 kD proteins of *A. stephensi* BBM. Dot-analysis revealed high activity of alkaline phosphatase in the extract of BBM proteins of *A. stephensi* larvae midgut. The concentrate of the fraction eluted from Cry11A-Sepharose at 1 M NaCl also exhibits activity of this enzyme. (Intensity of detected signal was low but significant.) Under these conditions of dot-analysis use of bovine serum albumin (0.2 mg/ml) as negative control was not accompanied by any color formation (data not shown).

DISCUSSION

Specificity of biological effects of BT endotoxins is determined by the presence (or absence) of toxin-binding proteins in insect gut epithelial membranes. Usually these proteins are defined in the literature as receptors [1]. This is not a perfect definition because such proteins do not belong to classes of protein receptors of any natural ligands. However, this definition well characterizes function of the toxin-binding proteins in the toxic effect and therefore it is widely used in the literature.

Receptors of Cry1 endotoxins are well described for caterpillar gut epithelium. They represent two groups of membrane proteins: aminopeptidases N and cadherin-like proteins [1, 9-11]. For various caterpillar species, more than 10 various aminopeptidases exhibiting toxin-binding activity have been identified and sequenced [1, 9, 10, 21, 22].

Little is known about receptors of toxins killing mosquito larvae (Cry4A, Cry4B, and Cry11A); however, immunohistochemistry revealed binding of activated Cry4B toxin and Cry11A endotoxin with gut epithelial apical membranes of these insects [23]. Recently we have isolated proteins from gut epithelium of *A. aegypti* larvae, which specifically bind some mosquitocide toxins [12, 13]. We have suggested that these proteins may function as receptors for endotoxins Cry4B and Cry11A in this mosquito species.

Using affinity chromatography on Cry4B- and Cry11-Sepharose, we have isolated 65 and 57 kD proteins from midgut epithelial membranes of *A. stephensi* mosquito larvae; these proteins can bind to endotoxin Cry11A and Cry4B-tox under conditions of ligand blotting (Figs. 1-3). Molecular masses of these proteins are similar to those of *A. aegypti* toxin-binding proteins (65 and 62 kD) [13]. Similar molecular masses have also been reported for *B. sphaericus* endotoxin receptor from gut epithelium of *Culex pipiens* mosquito larvae (60 kD) [24] and Cry1Ac-binding protein from *Heliothis virescens* caterpillar (68 kD) [25]. However, most lepidocide toxin receptors significantly differ in molecular mass, which varies from 100 to 170 kD in aminopeptidases N [1, 9, 10, 21, 22] and is 210 kD in cadherin-like proteins [11].

Our ligand-blotting binding experiments with 65 and 57 kD proteins isolated from *A. stephensi* larvae and Cry4B-tox and Cry11A revealed that: 1) the mosquitocide toxin binding to 65 and 57 kD proteins did not originate from their biotinylation because 10-fold excess of their non-biotinylated analogs prevented this binding (Fig. 3); 2) this binding was reversible (at least in the case of Cry4B-tox), because after incubation of nitrocellulose filters pretreated with the biotinylated toxin with excess of its non-biotinylated variant our detection system did not reveal any bands (Fig. 3); 3) experiments on homologous and heterologous competition of the mosquitocide proteins for binding with 65 and 57 kD proteins from *A. stephensi* (Fig. 3) show that Cry11A and Cry4B interact with the same proteins in membranes of this insect. Moreover, these toxins either bind at the same sites of the surface of these proteins or their binding sites are closely positioned to each other.

Similar results were obtained earlier for binding of Cry11A and Cry4B toxins with 65 and 62 kD proteins isolated from *A. aegypti* larvae [13]. This suggests certain similarity between toxin-binding proteins from gut membranes of the two mosquito species.

The toxin receptors isolated from various insect species usually do not bind Cry-proteins to which these species are resistant [10]. Consequently, binding specificity represents important evidence of involvement of the toxin-binding protein into receptor function *in vivo*. Under our experimental conditions of ligand-blotting the 65 and 57 kD proteins from *A. stephensi* did not react with Cry1A endotoxins (Cry1A-tox) exhibiting lepidocide activity and Cry3A endotoxin acting on beetle larvae [1] (Fig. 2). The Cyt1Aa endotoxin also did not bind to 65 and 57 kD proteins from *A. stephensi* (Fig. 2). This toxin exhibits high mosquitocide activity; however, it is suggested [1] that its toxic effect does not require interaction with a corresponding receptor. All these results suggest high specificity of interaction of 65 and 57 kD proteins from *A. stephensi* with BT toxins. Earlier we demonstrated lack of interaction of 65 and 62 kD toxin-binding proteins from *A. aegypti* membranes with Cry1Ab from ssp. *alesti* inactive against mosquito larvae [13].

The proteolytic pattern of Cry4B endotoxin depends on the specificity of the protease used (see the table). Chymotrypsin formed activated toxin of 64 kD corresponding to activated forms of BT toxins (Cry4B-tox) employed in the binding experiments described above. However, treatment with some other proteolytic enzymes resulted in hydrolysis of the actual toxin to a fragment of 50 kD (treatment with trypsin) (Cry4B-tr) or to fragments of 48 and 49 kD (treatment with *A. aegypti* gut juice) (Cry4B-Aa) [15]. In the first case, the active toxin lost five α-helices of the N-terminal domain, whereas in the second case small degradation also occurred in the C-terminal end of third domain. The tryptic product of endotoxin Cry4B proteolysis was even more toxic for *A. aegypti* than the activated toxin [15]; it also maintained binding activity with respect to *A. stephensi* 65 and 57 kD proteins (Fig. 2). However, the mixture of fragments of 49 and 48 kD was 20 times less toxic than the activated toxin [15] and it also failed to bind to the membrane proteins (Fig. 2). This is consistent with the modern notion on the involvement of the second and the third domains of the toxin molecule in receptor binding [1]. Limited proteolysis of endotoxin Cry11A yielding two fragments of 33 and 36 kD did not reduce its toxicity with respect to larvae *A. aegypti*, *A. stephensi*, and *Culex pipiens* [16]; such proteolytic treatment also did not influence its binding to *A. stephensi* membrane proteins (Fig. 2). It is possible that these "two halves" of the endotoxin Cry11A molecule are still pooled together forming an active complex, which may bind to corresponding receptor and perforate a sensitive membrane [16]. A similar correlation was also obtained during comparison of toxicity of proteolytic fragments of endotoxins Cry11A and Cry4B for *A. aegypti* larvae and binding of these fragments to 65 and 62 kD toxin-binding proteins from this insect [13].

Products of limited proteolysis of *Bacillus thuringiensis* endotoxins used in this study

Preparation	Initial endotoxin	Proteolysis	Protein composition, kD
Cry11A-tr	Cry11A	trypsin	33 and 36
Cry4B-tox	Cry4B	chymotrypsin	64
Cry4B-tr	Cry4B	trypsin	50
Cry4B-Aa	Cry4B	*A. aegypti* gut juice	49 and 48
Cry1A-tox	Cry1A endotoxins of B-9032	trypsin	66 and 61

Unfortunately, we failed to determine the N-terminal sequence of the 65 kD protein isolated from *A. stephensi* membranes. It is possible that the terminal amino group of this protein is modified. The 57 kD protein is characterized by a unique N-terminal sequence: we did not find such a sequence among sequences of the NCBI GenBank. Recently the full genome of *Anopheles gambiae*, a species related to *A. stephensi*, has been decoded and published. In the corresponding proteome, we also did not find a protein sequence, which would be identical to the sequence found in this study; nevertheless, this species has an open reading frame encoding the first six residues of our decapeptide (S-L-H-Q-H-I). Its accession number is EAL 38641. Although homology of 60% is quite reasonable for related proteins from various species, we perfectly understand that any conclusions made on the coincidence of small fragments of a polypeptide chain are not very convincing.

It was recently demonstrated that alkaline phosphatase (68 kD) is a putative endotoxin Cry1Ac receptor in the gut of *Heliothis virescens* caterpillar [25]. Using the method of dot-analysis, we also detected alkaline phosphatase activity in the fraction eluted from Cry11A-Sepharose. The staining was very weak but significant. Bovine serum albumin at a higher concentration as control did not cause any color development. The genome of *A. gambiae* contains genes encoding several proteins that might exhibit alkaline phosphatase activity. However, we did not find any similarity between the N-terminal fragment of 57 kD protein and these sequences. It is possible that alkaline phosphatase activity is associated with the 65 kD protein, but we could not determine its N-terminal sequence.

We cannot conclude that we have found all toxins binding proteins of *A. stephensi* membranes in the present study. Receptor proteins could loose affinity towards toxins during extraction from membranes (in this case the protein cannot bind to the affinity sorbents used) or during denaturation inevitably accompanying ligand-blotting (in this case we cannot detect a corresponding band in the eluate). Although known receptors of lepidocide toxins are always well tested by means of ligand-blotting [1], little is known about such properties of toxin-binding proteins from mosquito larvae.

Nevertheless, the bulk of the results obtained in this study suggests that the toxin-binding proteins which we isolated from two mosquito species, *A. stephensi* and *A. aegypti*, are related but possibly not identical. Perhaps they represent a new class (or classes) of receptors for entomocide proteins.

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