Insect Biochemistry and Molecular Biology xxx (2013) 1–10

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

# Interaction between mosquito-larvicidal *Lysinibacillus sphaericus* binary toxin components: Analysis of complex formation

Q1 Avinash Kale [b,1], Ramesh Hire [a,1], Ashok Hadapad [a], Stanislaus F. D'Souza [a,**], Vinay Kumar [c,*]

[a] Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai 400085, India
[b] High Pressure & Synchrotron Radiation Physics Division, Bhabha Atomic Research Centre, Mumbai 400085, India
[c] Solid State Physics Division, Bhabha Atomic Research Centre, Mumbai 400085, India

ARTICLE INFO

*Article history:*
Received 6 March 2013
Received in revised form
19 July 2013
Accepted 29 July 2013

*Keywords:*
Mosquito control
*Lysinibacillus sphaericus* binary toxin
BinA/BinB interactions
Covalent complex
Larvicidal activity

ABSTRACT

The two components (BinA and BinB) of *Lysinibacillus sphaericus* binary toxin together are highly toxic to *Culex* and *Anopheles* mosquito larvae, and have been employed world-wide to control mosquito borne diseases. Upon binding to the membrane receptor an oligomeric form (BinA2.BinB2) of the binary toxin is expected to play role in pore formation. It is not clear if these two proteins interact in solution as well, in the absence of receptor. The interactions between active forms of BinA and BinB polypeptides were probed in solution using size-exclusion chromatography, pull-down assay, surface plasmon resonance, circular dichroism, and by chemically crosslinking BinA and BinB components. We demonstrate that the two proteins interact weakly with first association and dissociation rate constants of $4.5 \times 10^3\ M^{-1}\ s^{-1}$ and $0.8\ s^{-1}$, resulting in conformational change, most likely, in toxic BinA protein that could kinetically favor membrane translocation of the active oligomer. The weak interactions between the two toxin components could be stabilized by glutaraldehyde crosslinking. The cross-linked complex, interestingly, showed maximal *Culex* larvicidal activity ($LC_{50}$ value of 1.59 ng $mL^{-1}$) reported so far for combination of BinA/BinB components, and thus is an attractive option for development of new bio-pesticides for control of mosquito borne vector diseases.



## 1. Introduction

Several isolates of *Lysinibacillus sphaericus* (previously known as *Bacillus sphaericus*) are highly potent against *Culex* and *Anopheles* mosquito larvae. These have been used as bio-pesticides for controlling vectors of human diseases like Nile fever, filariasis and malaria (Davidson, 1989; Oei et al., 1992; Berry, 2012). The highly active bacterial species produce binary (Bin) toxin, which accounts for the activity of sporulated cells, and toxins like sphaericolysin, and Mtx produced during vegetative growth (Berry, 2012). A parasporal crystal (inclusion) of Bin toxin is composed of two protoxins namely, pro-BinA (Mr, 41.9 kDa) and pro-BinB (Mr, 51.4 kDa) (Hindley and Berry, 1987; Baumann et al., 1988). Together these proteins are highly toxic to larvae of all *Culex* species and some species of *Anopheles*, while BinA alone shows reduced toxic activity against *Culex* (Oei et al., 1990; Nicolas et al., 1993; Hire et al., 2009). The *bin* genes are present only in a subset of *L. sphaericus* strains and sequences of the encoded proteins are highly conserved between strains, differing by no more than seven amino acids between any two variants (Priest et al., 1997; Humphreys and Berry, 1998; Hire et al., 2009).

After ingestion by the susceptible larvae the Bin inclusions are solubilized in alkaline midgut of the larvae, followed by activation of both pro-BinA and pro-BinB protoxins by larval proteases (Broadwell and Baumann, 1986; Davidson et al., 1987, 1990; Aly et al., 1989). The proteolysis of the protoxins occurs in both susceptible and non-susceptible insects (Nicolas et al., 1990). The deletion experiments have shown that 17 amino acids can be removed from both the N- and C-termini of BinA, while 34 residues can be removed from the N-terminus and 53 residues from the C-terminus of BinB without abolishing mosquito-larvicidal activity (Nicolas et al., 1990; Broadwell et al., 1990; Clark and Baumann, 1990; Oei et al., 1990). Also fusion proteins consisting of full-length BinA and BinB polypeptides were shown to be fully active in toxicity assays (Oei et al., 1990; Broadwell et al., 1990). The active

* Corresponding author. Tel.: +91 22 25593761; fax: +91 22 25505151.
** Corresponding author. Tel.: +91 22 25505342; fax: +91 22 25505151.
*E-mail addresses:* sfdsouza@barc.gov.in (S.F. D'Souza), vinay@barc.gov.in, vkbhatia@gmail.com (V. Kumar).
[1] Both the authors contributed equally.

0965-1748/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibmb.2013.07.011

BinA and BinB fragments from the polypeptide of the fusion construct were clearly seen in the western blot of the soluble proteins from the guts of larvae of *Culex pipiens* (Broadwell et al., 1990).

The active Bin toxin exerts toxicity by interacting with a GPI-anchored maltase receptor (Cqm1 in the susceptible *Culex* species) present on the midgut membrane of the host cell (Silva-Filha et al., 1999; Darboux et al., 2002; Opota et al., 2008). It is generally accepted that upon binding to the membrane an oligomeric form (BinA2.BinB2) of the binary toxin plays role in pore formation (Smith et al., 2005; Pauchet et al., 2005), similar to that reported for several binary bacterial toxins (Barth et al., 2004; Vachon et al., 2012). In the currently accepted model of *L. sphaericus* Bin toxin mode of action, the active oligomer is assembled on the receptor: BinB initially binds to the receptor and the active toxin component, BinA, interacts with the receptor bound BinB (Oei et al., 1992; Charles et al., 1997). The membrane insertion of competent oligomers subsequently leads to pore formation in the host cell and the oligomer is transformed inside the cell. Seemingly, BinA permeabilizes the membrane more efficiently than BinB (Schwartz et al., 2001). Also, presentation of Cqm1 receptor is essential for toxin induced pore formation, cytoplasmic vacuolation and autophagy in cultured mammalian epithelial MDCK cells (Pauchet et al., 2005; Opota et al., 2011).

*L. sphaericus* has proved to be a highly effective mosquito control agent in the field (Berry, 2012) despite the observed resistance that is mostly reported due to mutation in the receptor (Darboux et al., 2002, 2007; Silva-Filha et al., 2004; Romão et al., 2006). The amino acid sequence of toxin components may be a crucial factor for toxicity as well as *B. sphaericus* strains expressing orthologues of toxin components with no more than seven amino acids changes between any two variants differ dramatically in toxicity toward the susceptible mosquito strains. A commercial product (VectoLex®) based on *L. sphaericus* is marketed for the control of mosquito vectors of filariasis and viral diseases (Federici et al., 2003; Park et al., 2009). Several groups have attempted to identify *L. sphaericus* strains with higher toxicity toward different hosts (Hire et al., 2010; Hayes et al., 2011; Rashad et al., 2012). The best reported toxicity activity so far ($LC_{50}$, 1.7 ng $mL^{-1}$), however, has resulted from a recombinant bacterium that was engineered to express mosquitocidal Cry and Cyt proteins of *Bacillus thuringiensis* with the binary toxin of *L. sphaericus* (Federici et al., 2003). The recombinant Bti/BsBin also showed superior resistance characteristics such that significant resistance was not observed even after 30 successive generations of heavy selection pressure (Wirth et al., 2010). The *L. sphaericus* strains which are able to overcome the resistance have been observed to encode another two-component toxin consisting of Cry48Aa1/Cry49Aa1 proteins (Jones et al., 2007, 2008).

Apparently, *L. sphaericus* Bin toxin shares early events of toxin activity with *B. thuringiensis* crystal protein (Cry1Ab) toxin that interacts with specific cell receptors leading to the formation of insertion competent oligomers which in turn results in pore formation due to the insertion of oligomers in the membrane (Bravo et al., 2004; Vachon et al., 2012). In contrast to the monomers, the purified Cry1Ab oligomer more efficiently inserts in the membrane producing stable and open channels (Rausell et al., 2004; Pardo-López et al., 2006). The *L. sphaericus* Bin protoxins have also been suggested to oligomerize in solution to form BinA2.-BinB2 complex, but trypsin-digested BinA and BinB, those resembling active forms of the proteins, did not form such oligomers (Smith et al., 2005). However, formation of stable heteromeric complex of active proteins has recently been suggested using low-resolution size-exclusion column chromatography (Srisucharitpanit et al., 2012).

We used several techniques to probe stable or transient interactions between BinA and BinB proteins from a highly toxic strain of *L. sphaericus*. We did not find evidence of stable BinA2.-BinB2 tetramer, though weak interactions resulting in conformational change in BinA were detected by surface plasmon resonance and circular dichroism. Intriguingly, a covalent complex of binary components, synthesized by crosslinking active BinA and BinB peptides with glutaraldehyde, showed significant improvement in toxicity against third-instar larvae of *Culex quinquefasciatus*.

## 2. Materials and methods

### 2.1. Materials

The local strain of *L. sphaericus* (*B. sphaericus* ISPC-8) having serotype 5a5b and phage type 3 was that reported earlier (Hire et al., 2009). dNTPs were obtained from Roche, Germany, restriction enzymes from New England BioLabs, and Phusion high fidelity DNA polymerase was obtained from Finnzyme. *Escherichia coli* BL21(DE3) [*E. coli* B F − *dcm ompT hsdS* ($r_B^-$, $m_B^-$) *gal*λ (DE3)], and *p*ET28a were from Novagen. Chromatography media and prepacked columns were obtained from Bio-Rad and GE Healthcare. Oligonucleotides for cloning were synthesized at BRIT, India. The other fine chemicals were obtained from SRL, India.

### 2.2. Cloning of *binA* and *binB* genes

The primers specific for *bin*A gene were designed to express active BinA protein lacking 15 residues from the N-terminus and 4 residues from the C-terminus of the BinA protoxin based on the *bin*A gene sequence (GenBank accession number EU375308) of *L. sphaericus* ISPC-8. The forward and reverse primers for *bin*A amplification were 5′-ATACCCACACATATGAAGTACATTCGC-3′ and 5′-GTTGGATCCTTATGTAATAATCTTTG-3′ respectively (the NdeI and BamHI restriction sites are underlined). The 1056 bp *bin*A coding sequence was PCR amplified using these primers from the genomic DNA of *L. sphaericus* ISPC-8 (Hire et al., 2009), and Phusion high fidelity DNA polymerase (Finnzyme). The PCR amplified *bin*A coding sequence was digested with NdeI and BamHI, and ligated in the same site of *p*ET28a (*p*ET28-*bin*A) construct.

The primers specific for *bin*B gene were designed based on the sequences available in GenBank (accession numbers AJ224477, AJ224478 and DQ875600). The forward and reverse primers for *bin*B amplification were 5′-GATGAAGAACATATGTGCGATTCAAAA-GAC-3′ and 5′-AGTTGGATCCTTACTGGTTAATTTTAGGTATTAA-3′, respectively. The 1347 bp *bin*B coding sequence was PCR amplified using these primers from the genomic DNA of *L. sphaericus* ISPC-8. The PCR amplified *bin*B coding sequence was digested with NdeI and BamHI, and ligated in the same site of *p*ET28a (*p*ET28a-*bin*B construct).

Cloned *bin*A and *bin*B gene constructs were individually transformed in *E. coli* DH5α for verification and plasmid purification, while *E. coli* BL21(DE3) was used for protein expression and purification. The nucleotide sequences of the *p*ET28a-*bin*A and *p*ET28a-*bin*B constructs were confirmed by complete sequencing of *bin*A and *bin*B genes using an ABI automated DNA sequencer. These sequences matched exactly with chromosomal gene sequences (GenBank accession nos. EU375308 and EU375309). The deduced amino acid sequence of the expressed BinB protein matched with highly toxic *L. sphaericus* strains 1593 and C3–41 except for the H109P and P274S changes, while BinA sequence showed only M197R substitution as compared to the sequences from *L. sphaericus* strains 1593 and C3–41.



Please cite this article in press as: Kale, A., et al., Interaction between mosquito-larvicidal *Lysinibacillus sphaericus* binary toxin components: Analysis of complex formation, Insect Biochemistry and Molecular Biology (2013), http://dx.doi.org/10.1016/j.ibmb.2013.07.011