

Phage display of *Bacillus thuringiensis* CryIA(a) insecticidal toxin

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Received 14 May 1997

Abstract The display of proteins or peptides on the surface of filamentous phages or phagemids has been shown to be a very powerful technology for the rescue of specific binders from large combinatorial libraries, as well as to select derivatives of known proteins with altered binding properties. The *Bacillus thuringiensis* (Bt) crystal proteins are a large family of insecticidal toxins which bind to receptors found on the brush border of larval midgut cells, different crystal toxins having different larval specificities. Here we describe the display of different CryIA(a) toxin regions on the surface of phagemids using the display vector pHEN1, the purpose being the identification of toxin sequences suitable for mutagenesis and selection using phage display. We show that CryIA(a) domain II, in which the receptor binding activity is located, is efficiently displayed as well as being secreted as soluble protein into the periplasm of bacterial cell. This forms the basis of a simple means for the modification of toxin specificity and the selection of toxin proteins with novel or expanded host ranges.

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Key words: Phage display; Phagemid; δ -Endotoxin; Insecticidal crystal protein

1. Introduction

Different isolates of soil *Bacillus thuringiensis* produce parasporal crystals. These crystals are composed of δ -endotoxins which are toxic to some insect pests and disease vectors. To date more than 50 different *cry* genes have been sequenced and reported. The sequence homology of different *cry* genes and analysis of the X-ray structure of CryIIIa [1] and CryIA(a) [2] has revealed certain common features. Each consists of three distinct domains. Domain I spans amino acid residues 1–290 and is believed to play an important role in the formation of the pore in the target cell membrane, this being considered to cause the toxicity of the protein. Hypervariable domain II, spanning amino acid residues 291–500 has been implicated in binding to the insect cell surface and can be considered to encode the receptor binding part of the toxin. Domain III, from residues 501 to C-terminus, has been speculated to play a role in toxin stability and toxin integrity. Thus, interaction of domain II with the mid-gut receptor is considered one of the key factors for insect toxicity and specificity. Cry proteins which do not have measurable receptor binding activity are usually not toxic. Shuffling/swapping of domain II among different genes has resulted in alteration of insect susceptibility profile in a predictable fashion [3,4]. Similarly,

point mutations in the domain II can lead to the loss of toxicity, towards target pests.

Geometrically, domain II displays three surface exposed loops. Of these, residues RRPFNIGI within loop 2 have been postulated to play an important role in the molecular recognition process of toxin-receptor interaction [5]. In addition to the identification of critical amino acids involved in receptor recognition in domain II, $\alpha 5$ segment of domain I has also been evaluated for pore formation ability [6]. These observations raise the possibility that local amino acid substitutions or mutations within the critical regions of domain II may be a means to increase toxin host range and potency. We believe peptides of domain II and I could be exploited to simulate the interaction of the toxin with its receptor on the susceptible insect cell surface and the subsequent toxicity.

Recently, filamentous bacteriophages have been used to display peptides and proteins fused to the minor g3p or major g8p coat proteins [7,8]. Libraries of mutant or variant proteins can be cloned into phage proteins (usually g3p or g8p) by a number of different mutagenesis techniques. After rounds of selection, each of which involves the binding of phages to an appropriate ligand, elution and amplification, variants of the original protein can be derived on the basis of modified binding activity. Two vector systems have been used for the creation of such libraries. Phagemids are plasmids which contain a phage origin of replication as well as either *gene III* or *gene VIII* which are modified to allow the insertion of exogenous sequences [9]. These require the use of helper phages (which supply all the others phage proteins necessary to make functional phage) to rescue the fusion protein which is incorporated into phage particle along with the helper phage g3p. In phage vectors, *gene III* or *VIII* is directly modified by the insertion of the exogenous sequences and are functional in the absence of helper phages. In the phagemid pHEN1 a myc tag and an amber stop codon are present at the 5' end of *gene III*. The amber mutation allows the secretion of the cloned polypeptide in addition to its display, while myc tag is recognized by monoclonal antibody (mAb) 9E10 [10]. Here we describe the cloning of different CryIA(a) toxin fragments into the pHEN1 phagemid vector and report the expression of the fusion proteins on the phage coat. The final goal is to obtain a phagemid library expressing different toxin specificities which can be selected on receptors of resistant insect species.

2. Materials and methods

2.1. PCR and cloning

The fusion proteins between fragments of CryIA(a) protein and glutathione S-transferase (GST) or *gene III* were constructed by PCR using a pBluescript KS+ (Stratagene) plasmid containing a

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cloned Bt *CryIA(a)* gene as template or by PCR assembly of oligonucleotides. The following primers were used:

Bt1 5'-AGGCTGCAGGCTAGGAACCAAGCCATTTC-3'
 Bt2 5'-CGGCTCGAGAAAAATCCCCAACCAAGTTA-3'
 Bt3 5'-GGTCTCGAGATACGTTGGATAGCACTCAT-3'
 BtL2-1 5'-CGCGGATCCTGCAGGATTATAAAGATGAAGAA-
 GAATTATACTTGGT-3'
 BtL2-2 5'-CCGGAATTCTCGAGTTTCCTGATTATTTGGGCCT-
 GAACCAAGTATAA-3'
 BtDII-1 5'-CGCGGATCCTGCAGTTTGATGGTAGTTTTTCGTG-
 GA-3'
 BtDII-2 5'-CCGGAATTCTCGAGACTGCGATGCTGCCAA-
 GAAA-3'

Enzyme restriction sites are underlined; the primer Bt1 contains a *Pst*I site; the primers Bt2 and Bt3 contain an *Xho*I site; two overlapping sites were inserted in the remaining primers: *Bam*HI and *Pst*I in BtL2-1 and BtDII-1 and *Eco*RI and *Xho*I in BtL2-2 and BtDII-2. Bold sequences correspond to Bt *CryIA(a)* gene. The primer BtL1 contains the sequence for FLAG tag sequence (in italic). Three fragments of *CryIA(a)* gene overlapping regions were amplified by PCR (Fig. 1). The fragments coding amino acids 92–358 (BtS – short fragment) and amino acids 92–732 (BtL – large fragment) of the native protoxin were amplified using Bt1 as sense primer and Bt2 and Bt3 as reverse primers, respectively. The fragment encoding domain II (BtDII, amino acids 276–458) was amplified with BtDII-1 as sense and BtDII-2 as reverse primer. The PCR conditions were: 30 cycles of 94°C 1 min, 55°C 1 min, 72°C, 2 min. The primary PCR amplification products were gel purified and digested with *Pst*I and *Xho*I to subclone in pHEN1 phagemid vector. The resulted vectors were named pHEN1–BtL, pHEN1–BtS and pHEN1–BtDII. The amplified fragment BtDII was digested with *Bam*HI and *Eco*RI; since the domain II sequence contains an *Eco*RI site the digestion generated two fragments; these were consecutively cloned in the pGEX-3X vector (Pharmacia Biotech) to obtain the vector pGEX–BtDII.

To generate fragment BtL2 coding for amino acids from 367 to 379, a PCR with overlapping oligonucleotides BtL2-1 and BtL2-2 was performed without template. PCR conditions were: 40 cycles of 94°C 1 min, 50°C 1 min, 72°C 2 min. The PCR product of the correct dimension was gel purified and digested with *Pst*I and *Xho*I to subclone in pHEN1 phagemid vector, and with *Bam*HI and *Eco*RI to subclone in the pGEX-3X vector. The subsequent vectors were named pHEN1–BtL2 and pGEX–BtL2, respectively. Following purification, aliquots of all recombinant vectors were used to transform *E. coli* DH5αF'. Individual colonies were screened for correct plasmid insertion by PCR and by DNA restriction analysis.

2.2. Phage preparation

To 1 ml of phagemid bearing bacteria (OD⁵⁵⁰ 0.5) 10¹² pfu (plaque forming units) M13 K07 helper phage were added. After 30 min incubation at 37°C the bacteria were centrifuged and resuspended in Luria Bertani (LB) medium containing 100 µg/ml ampicillin and 20 µg/ml kanamycin. After an overnight growth, phages were prepared from supernatant by two 20% PEG (20% polyethylene glycol 6000, 2.5 M NaCl) precipitation. The phage pellet was resuspended in a volume of distilled water, 1:100 of the original culture volume. Phage titre was determined by making serial dilutions of phage suspension and adding 1 ml of DH5αF' bacteria (OD⁵⁵⁰ 0.5). Drops of 5 µl of serially diluted bacterial suspension were plated on LB ampicillin-containing medium. After an overnight growth the number of ampicillin-resistant colonies was counted.

2.3. Defective helper phage

In one experiment the non-infectious mutant phage fKN16 was used. fKN16 carries a deletion removing the amino acids 29–197 of g3p [11]. DH5αF' cells bearing fKN16 were transformed with phagemid DNA and selected on solid medium containing ampicillin. Phage particles were collected from supernatant as described above.

2.4. pGEX–BtD2 and pGEX–BtL2 expression

The vectors pGEX–BtDII and pGEX–BtL2 were used to express fusion protein GST–BtDII and GST–BtL2 in *E. coli*. After transfor-

mation, clones were screened by PCR and the correct orientation was checked by expression of the fusion protein. Positive clones were grown and after induction with 1 mM IPTG for 3 h, bacteria were pelleted and lysed. The fusion proteins were purified by binding to glutathione–agarose (GS4B, Pharmacia) and elution with reduced 15 mM glutathione according to manufacturer's instructions. Finally, total cell lysates and soluble fusion proteins bound to glutathione–agarose were analyzed by SDS PAGE.

2.5. Preparation of rabbit antiserum to GST–BtL2 and affinity purification of antibodies

One rabbit was injected with 300 mg of purified bacterial GST–BtL2 fusion protein mixed 1:1 with complete Freund's adjuvant. This was followed by two booster shots of the same amount of protein in incomplete adjuvant. Immune serum (2 ml), after preclearing on GST column, was affinity purified by binding to Affi-Prep 10 support (Bio-Rad Laboratories) coupled to 1 mg of fusion protein, according to manufacturer's instructions.

2.6. SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate (SDS) electrophoresis was performed according to standard techniques. 10¹² cfu (colony forming units) phagemids were denatured in boiling loading buffer, separated by polyacrylamide gel electrophoresis and transferred onto nitrocellulose (Amersham) by semi-dry blotting using the Pharmacia Multiphor II. The membrane was blocked using 5% skimmed milk in PBS (MPBS) for 1 h at room temperature. A mAb to g3p [12], a mAb to myc tag or rabbit antibodies to GST–BtL2 were used as primary antibody. Myeloma culture supernatants containing the mAbs were diluted 1:5 in MPBS, purified anti-GST–BtL2 antibodies were diluted 1 µg/ml in MPBS and added to the blotted nitrocellulose sheets. After an overnight incubation at 4°C and extensive washing with PBS plus 0.05% Tween 20, the nitrocellulose was subsequently incubated with anti-mouse IgG goat antibodies conjugated with alkaline phosphatase or peroxidase (Dako), or anti-rabbit IgG goat antibodies conjugated with peroxidase (Dako). The positive bands were revealed by the chromogenic substrate BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) (for alkaline phosphatase) or chemiluminescent ECL (Amersham) system (peroxidase). In one case a mAb M1 (Kodak) anti-FLAG was used as primary antibody according to the protocol in [13].

3. Results

3.1. Cloning strategy

The cloning strategy is reported in Fig. 1. We have cloned four different *CryIA(a)* regions corresponding to almost the entire activated toxin, reported here as BtL, the N-terminal domain (BtS), the domain II (BtDII) and the second loop found in domain II RRILGSGPNNQE (BtL2).

3.2. Cloning and expression of BtL and BtS

BtL and BtS sequences were obtained by PCR amplification of *CryIA(a)* cloned genes. After cloning into pHEN1, individual colonies were grown in liquid medium and infected with M13K07 helper phage. Following infection, bacteria bearing pHEN1–BtL lysed within 30 min while BtS showed slower growth than controls. pHEN1–BtL bacteria titrated before and after phage infection showed a loss of viability of around 90%. No induced mortality was registered in pHEN1–BtS cells. After overnight growth, phagemids were recovered from the supernatant by PEG precipitation and titrated. The titres of the phage produced by viable pHEN1–BtL bacteria and pHEN1–BtS were always considerably less than pHEN1 controls (Table 1). In Western blots, no band with a molecular weight higher than g3p was recognized by the anti-g3p mAb (not shown). Considering the possible toxic effect of the recombinant g3p, infected bacteria were grown in presence of 1% glucose to reduce the expression of

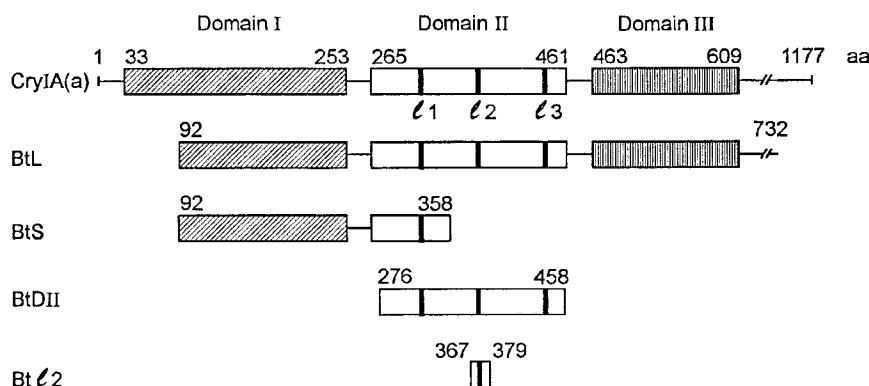


Fig. 1. Schematic drawing of the cloning of different CryIA(a) toxin regions. Reference numbers of the amino acid residues at the ends of the cloned sequences are shown. The vertical bars in domain II represent three structural loops.

the cloned toxin (glucose reduces the activity of the lac promoter controlling expression of the displayed protein g3p fusion). Bacteria grown under these conditions gave a slightly higher phage titre (Table 1), and Western blot of 10^{12} phages grown under these conditions showed a faint band with an apparent molecular weight of 90 kDa corresponding to the expected size of the fusion protein as well as a strong band of 70 kDa corresponding either to the helper phage g3p or partially degraded recombinant g3p (Fig. 2, lane 2, arrows B and A, respectively). In Fig. 2, lane 1, a reference pHEN1 phage is reported. Growth in glucose did not improve the display of BtL. To further improve the incorporation of the recombinant protein, a defective helper phage, fKN16, was used. This phage has a deletion in *gene III* and is non-infectious unless it incorporates g3p provided by a source in trans. In a previous experiment we found that fKN16 acts as helper phage utilizing preferentially the recombinant g3p instead of its partially deleted g3p. DH5 α F' bacterial cells harbouring fKN16 were transformed with pHEN1–BtL phagemid DNA and, after growth, phages were recovered from the supernatant. The phage titre is reported in Table 1 and the respective Western blot of 10^{12} cfu in Fig. 2, lane 3. In this case, other than the low molecular weight g3p of fKN16 (arrow D), an abundant band in the range of g3p (arrow A) and an additional band around 94 kDa (arrow C) are recognized. A similar pattern is revealed by the bacterial cells producing the phagemids (Fig. 2, lane 4). Our interpretation of these results is that the recombinant protein, with a nominal molecular weight of 150 kDa, is degraded to band C and from there to a protease resistant g3p core. The last is selectively incorporated by nascent phages resulting in a limited presence of fragment C on the phage surface.

Table 1
Titres of phages produced by bacteria harbouring phagemids recombined with different CryIA(a) sequences

Phagemid	Helper phage	Medium	cfu/ml of culture
pHEN1	M13 K07	LB	10^{13}
pHEN1–BtS	M13 K07	LB	5×10^9
pHEN1–BtL	M13 K07	LB	5×10^8
pHEN1–BtS	M13 K07	LB+1% Glu	10^{10}
pHEN1–BtL	M13 K07	LB+1% Glu	10^9
pHEN1–BtL	fKN16	LB	10^{12}
pHEN1–BtL2	M13 K07	LB	10^{13}
pHEN1–BtDII	M13 K07	LB	10^{13}

3.3. Cloning and expression of BtDII and BtL2

Loop 2 RRIILGSGPNNQE fragment was obtained by PCR assembly of two oligonucleotides comprising the coding sequence, the restriction sites for the insertion in pHEN1 and pGEX and the coding sequence for the FLAG epitope DYKDE recognized by the mAb M1. This tag was inserted at the N-terminus to facilitate the recognition of the loop 2 peptide on g3p. DII fragment was obtained directly by PCR amplification of CryIA(a) cloned gene. After transformation the positive clones were identified by PCR. The titres of the resulting pHEN1–BtL2 and pHEN1–BtDII phages, reported in Table 1, are similar to those of the control pHEN1 phage, indicating that these constructs are far less toxic than pHEN1–BtS and pHEN1–BtL phagemids. In Western blotting using the mAb anti-g3p, 10^{12} cfu of pHEN1–BtDII phages (Fig. 3, lane 3) show a doublet of 85/90 kDa (arrow A) in addition to a g3p sized band. 90 kDa is the size expected for full-length domain II attached to g3p, indicating that this domain is well displayed. The smaller 85 kDa band probably represents a degradation product which has lost approximately 50 amino acids at the N-terminus. The g3p band is a doublet, probably due to the difference in molecular weight between the g3p of the helper phage and the g3p of pHEN1 which has an additional polylinker and a myc tag sequence. According to this interpretation, the double g3p band present in pHEN1–BtDII attests a partial degradation of the fusion protein. As far as the pHEN1–BtL2 is concerned, no evidence of expression can be inferred by Western blot (Fig. 3, lane 2) because the reduced dimension of the peptide. Unfortunately, no recognition occurred using the anti-FLAG mAb. We hypothesize that the mAb failure in tag recognition could be caused by the toxin amino acids positioned downstream. It has been demonstrated that the type of the amino acid following the FLAG peptide disturbs the mAb reactivity [14]. To confirm the expression of the cloned peptide on the phagemid particles, a Western blot using a rabbit antiserum raised to GST–BtL2 fusion protein (see below) was performed. The results are reported in Fig. 3. If compared to the pHEN1 control (lane 4) the pHEN1–BtL2 phages show a strong positive band (lane 5) corresponding to the fusion protein BtL2–g3p.

The ability of pHEN1–BtDII phages to direct the secretion of domain II fragment was investigated in a non-suppressor *E. coli* strain (HB2151). In these bacteria the amber codon is read as a stop codon causing the release of the cloned protein into the periplasmic space. The Western blot of the periplas-

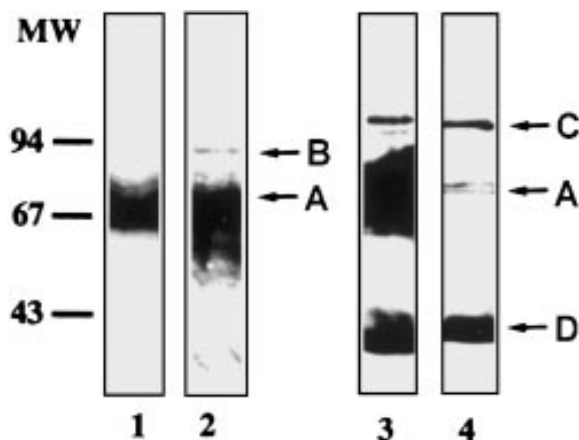


Fig. 2. Western blots of phages displaying CryIA(a) toxin fragments. Lane 1: control pHEN1; lane 2: pHEN1-BtS; lane 3: pHEN1-BtL (fKN16 helper phage), lane 4: protein extract of bacterial cells harbouring pHEN1-BtL and fKN16. Lanes 1 and 2: anti-g3p monoclonal antibody; secondary antibody: alkaline phosphatase-conjugated goat anti-mouse antibodies; substrates: BCIP and NBT. Lanes 3 and 4: mAb anti-g3p; secondary antibody: peroxidase-conjugated goat anti-mouse antibodies. Substrate: ECL. Arrow A: g3p; arrow B: BtS-g3p fusion protein; arrow C: proteolytic fragment of BtL-g3p; arrow D: fKN16 deleted g3p.

mic space of HB2151 *E. coli* infected with pHEN1-BtDII phages is shown in Fig. 3, lane 7. The BtDII band identified by mAb 9E10 recognizing the myc tag at the C-terminus has an apparent molecular weight of around 25 kDa, corresponding to that expected for the size of domain II. In lane 6 pHEN1 phagemids are reported for reference.

3.4. Cloning and expression in pGEX

BtDII and BtI2 sequences were cloned in pGEX which will express the cloned gene as a protein fused to glutathione S-transferase. The purpose of the cloning was to study the expression of the toxin fragment in another fusion system and provide purified toxin antigens for the production of specific antisera. After cloning, bacterial cell lysates were purified by affinity chromatography and the eluted fractions analyzed by

SDS-PAGE. In Fig. 4 the cell lysate (lane 1), the purified GST-BtDII (lane 2) and GST-BtI2 (lane 3) are reported. The mobility of GST-BtDII polypeptides corresponds to that expected for the predicted molecular weight of the fusion product. The GST-BtI2 band has a mobility slightly lesser than the GST polypeptide alone (lane 4) attesting the expression of the BtI2 peptide. The polypeptides bands are clean and without degradation products. Purified GST-BtI2 was used to obtain the rabbit antiserum mentioned above.

4. Discussion

To date many different molecules have been used for phage display, including antibody fragments, enzymes, and hormones [15]. The possibility of using phage display to isolate a polypeptide with binding characteristics which differ from the original protein depends on the level of expression and stability of the displayed molecule. Here we have displayed different parts of the CryIA(a) toxin in an attempt to understand the form most suitable for display and subsequent mutagenesis and selection. Cloning of a large fragment corresponding to the activated toxin caused slowed bacterial growth, but did not cause lysis unless the bacteria were also infected with a helper phage. We feel that this may arise from the insertion of a functional toxin pore molecule into the cell membrane following phage extrusion. The induction of toxicity is probably responsible for the lack of display observed. Using the defective phage fKN16 we found that a low amount of the toxin could be incorporated, but the dimension of the polypeptide is far lower than the whole toxin and presumably lacking the N-terminal region where the lytic activity is located. In a recent work [16] it is shown that mutations in conserved block 4 of domain III affect conductance of ion channels formed by the toxin. This finding may account for the high lysis with pHEN1-BtL as compared to pHEN1-BtS. A better result is obtained cloning loop 2 binding region and domain II sequences. Bacteria bearing respective recombinant phagemids showed no problems during growth and rescued phages had normal titres. Since the mAb anti-FLAG failed the epitope recognition, the expression of the loop 2 sequence

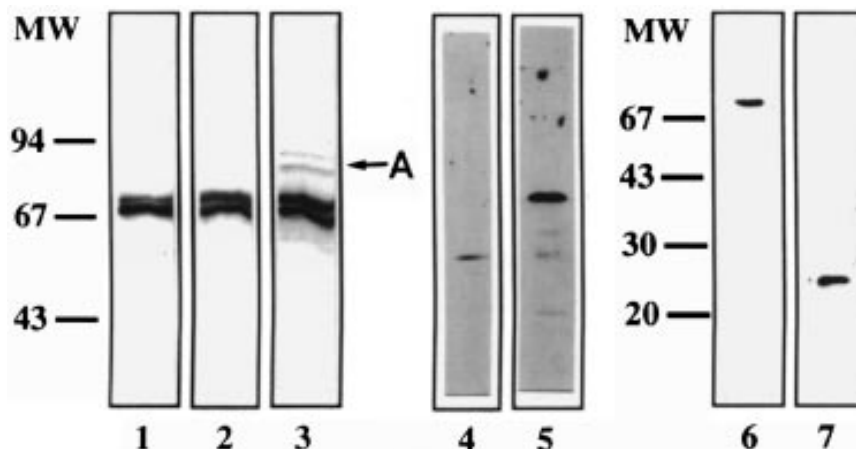


Fig. 3. Western blots of phages displaying CryIA(a) toxin fragments and soluble toxin. Lane 1: control pHEN1; lane 2: pHEN1-BtI2; lane 3: pHEN1-BtDII; lane 4: control pHEN1; lane 5: pHEN1-BtI2; lane 6: control pHEN1; lane 7: soluble BtDII. Lanes 1–3: mAb anti-g3p; secondary antibody: alkaline phosphatase-conjugated goat anti-mouse antibodies. Substrates: BCIP and NBT. Lanes 4 and 5: affinity purified rabbit antibodies anti BtI2; secondary antibody: peroxidase-conjugated goat anti-rabbit antibodies; substrate: ECL. Lanes 6 and 7: anti-myc tag monoclonal antibody; secondary antibody: peroxidase-conjugated goat anti-mouse antibodies. Substrate: ECL. Arrow A indicates BtDII-g3p fusion protein bands.

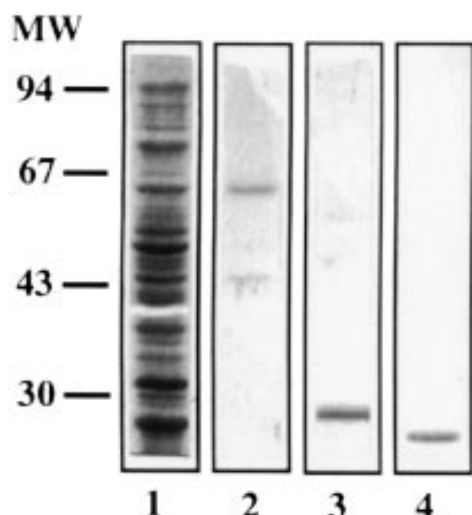


Fig. 4. Coomassie Blue-stained SDS-PAGE of CryIA(a) toxin fragments cloned in pGEX plasmid. Lane 1: bacterial extract; lane 2: affinity purified GST-BtDII; lane 3: purified GST-BtI2; lane 4: purified GST.

on phages was confirmed by the antiserum specifically raised to the GST-BtI2 fusion protein. On the basis of Western blot band intensity, we estimate that domain II is displayed on approximately 5–10% of phagemids, a level comparable to those we see with other proteins, such as single chain antibody fragments (data not shown). The correct synthesis of domain II is confirmed by the purity of the soluble form secreted by non suppressor *E. coli* strain. Both BtI2 and BtDII yielded large amount of undegraded fusion proteins when cloned in pGEX vector which expresses these polypeptides fused to GST.

In conclusion loop 2 and domain II appear promising for phage binding studies. In particular, for the creation of libraries of mutants with changes in amino acids predicted to be important for binding to the target cell receptor. Such phage libraries could be panned to isolate new toxin specificities to resistant insect species on the basis of phage binding to mid-gut vesicles, cultured cells [17] or cell receptors cloned and

expressed as fusion protein on the surface of bacterial cells [18] or as purified proteins.

Acknowledgements: We gratefully acknowledge Dr. G. Winter and Dr. H. Hoogenboom for plasmid pHEN1; Dr. M. Tesar (GBF, Braunschweig) for mAb anti-g3p. We also thank N. Arora for his excellent technical help.

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