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Bacillus thuringiensis Vip3Aa1 Expression and Purification from *E. coli* to be Determined in Seeds and Leaves of Genetically-Modified Corn Plants

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Abstract: Alternatives to *Bacillus thuringiensis* (Bt) pesticide crystal protein (Cry), such as vegetative phase insecticidal proteins (Vip), to combat insect in genetically-modified plants has being investigated to provide an insect resistance management additional tool. Likewise, this study aimed expressing and purifying Bt-Vip3Aa1 from an *Escherichia coli* strain for generating polyclonal rabbit antibodies to be then employed for Vip3Aa1 detection in genetically-transformed corn plants. To perform this subject, Vip3Aa1 was expressed in the JM-109 strain and purified by metal-chelate affinity chromatography. Next, a Dot-blot, Western-blot and an enzyme-linked immunosorbent assay (ELISA) using Vip3Aa1-specific polyclonal rabbit antibodies were carried out to detect Vip3Aa1 in genetically-modified corn plant seeds and leaves, respectively. FR-Bt1 corn plants transformed with Cry1Fa1 gene served as negative control. As results, Vip3Aa1 production method yielded up to 16 μg of purified Vip3Aa1 per fermentation supernatant milliliter, 50.4±4.0% recovery and 97.2±2.7% of SDS-PAGE purity. Vip3Aa1 amino acid sequence corresponded totally with expected sequence and Vip3Aa1 shown a high insecticidal (93.5±8.8%) at 2 μg mL^{-1} and immunogenicity capacity in rabbits (1:30000±4142). Vip3Aa1 quantification assays evidenced high specificity and sensitivity (1 ng mL^{-1} (Dot-blot) and 1 ng mL^{-1} (detection limit) 30 ng mL^{-1} (quantification limit)) in the ELISA. Quantification results were further corroborated through a biological assay using *Spodoptera frugiperda* and plant materials as insect diet supplements, where death rate ranged 68.7-7.0% in a doses dependent manner. Thus, these immunoassays can be successfully used to detect Vip3Aa1, in the seeds and leaves allowing discrimination between modified-and non-modified-genetically corn seeds and plants.

Key words: *Bacillus thuringiensis*, genetically-modified corn plants, vegetative phase insecticidal proteins, Vip3Aa1

INTRODUCTION

Bacillus thuringiensis (Bt) is one of the bio-pesticide worldwide leaders (Pardo-Lopez *et al*., 2009; Li *et al*., 2008; Liu *et al*., 2008) and among its pathogenic factors, crystal proteins (Cry) and Cyt δ-endotoxins are the most relevant (Bravo, 1997; Schnepf *et al*., 1998; De Maagd *et al*., 2003; James, 2007; Sohail *et al*., 2012; Karimi *et al*., 2013). In this sense, genes encoding Cry toxins have been genetically employed to generate transgenic insect-resistant crops (James, 2007; Clive, 2012; Sohail *et al*., 2012; Karimi *et al*., 2013). However, there is a growing concern about insect-resistance by the extended and prolonged use of these genetically-modified crops (Kain *et al*., 2004).

As consequence; proteins with a different insecticide action mode to Cry and Cyt δ-endotoxins are attractive for insect resistant transgenic crop generation (Fang *et al*., 2007). For instance, vegetative phase insecticidal proteins (Vip) such as (Vip1 and Vip2 binary toxins) and Vip3 have been examined as an alternative to induce beetles and *Lepidopteran* species resistance in plants, respectively (Estruch *et al*., 1996; Rang *et al*., 2005).

In particular, Vip3Aa1 has been recently stacked with others Cry toxins in different commercial transgenic events of corn and cotton to expand the targeted pest range and for a proper insect resistance management. In details, it is highly insecticide against *Spodoptera frugiperda*, a plague that affects corn crops in Cuba and other countries of the Caribbean region and Western hemisphere (James, 2007; Clive, 2012).

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In regards to its action mode, this toxin causes middle bowel cell lysis via pore formation. Nevertheless, this mechanism has been not totally well elucidated yet. Reasons why, researchers speculate that Vip3Aa1 action mode is different from that observed in Cry toxins (Lee *et al*., 2003). Despite this, the use of genetically-modified plants to express Vip3Aa1 is also a great concern in terms of insect-resistance.

On the other hand, the use of antibody-based immunoassays for the detection of transgenic proteins in multiples applications has also been broadly reported (Fantozzi *et al*., 2007; Wang *et al*., 2007; Jang *et al*., 2011). In this regards, there are commercially available immunoassays for Cry and Vip protein detection (Adgia Inc., Desigen; EnviroLogix Inc., Strategic Diagnostics Inc.). But, to have own Vip3Aa1 quantification systems for screening of a large number of samples is something highly desired.

Overweighting or due to the reasons mentioned in this introductory information, this study aimed expressing and purifying Vip3Aa1 from a genetically-transformed *Escherichia coli* strain for generating polyclonal rabbit antibodies that could be then employed for Vip3Aa1 detection in genetically-transformed corn plants.

MATERIALS AND METHODS

Vip3Aa1 gen cloning and expression: The gene encoding Vip3Aa1 was amplified from the AB88 Bt strain using 5'-GGATCCATGAACAAGAATAATACTAAATTAA GCAC-3' and 5'-GGTACCTTACTTAATAG AG ACAT CGTAAAAATG-3' primers. For recombinant expression, Vip3Aa1 DNA fragment was attached into restriction BamHI/Kpnl sites of pQE-30 expression vector and resultant plasmid pQE-Vip introduced into the *Escherichia coli* JM-109 strain (Invitrogen, Carlsbad, CA, USA) by a thermic shock transformation procedure. All reagents used were supplied by Promega (Madison, USA).

Vip3Aa1 transformed JM-109 strain cultivation: A transformed strain colony was inoculated into 50 mL of Luria-Bertani (LB) medium composed by: yeast extract (5 g L^{-1}), tryptone (10 g L^{-1}) and NaCl (10 g L^{-1}), while Ampicillin (0.05 g L^{-1}) was used as selective antibiotic. The primary culture was incubated at 37°C for 15 h with gentle stirring. After that, 125 mL of grown culture were inoculated into 300 mL-Erlenmeyer flasks or a 5L-fermenter (B.E. Marubishi, Tokyo, Japan) with LB medium and incubated at 37°C at 350 rpm for others 17 h. Absorbance was measured at 600 nm to estimate optimal time for the induction of Vip3Aa1 expression with 1 mM IPTG (~3 h). After inductor addition, culture was incubated again at 28°C for 15 h.

Vip3Aa1 purification

JM-109 strain disruption: Cell culture was centrifuged at 3300 g for 30 min at 4°C and pellet obtained was solubilized in 10 mM Tris-HCl, 20 mM NaCl; pH = 8.0 and a protease cocktail (Promega, Madison, USA). Next, transformed cells were broken using a French press at 1000-1500 kpa during two disruption cycles. The disruption suspension was centrifuged at 31000 g for 30 min and supernatant was stored at 4°C until Vip3Aa1 purification.

Metal-chelate affinity chromatography (IMAC) and size-exclusion chromatography procedures: The IMAC matrix (GE Healthcare Lifescience, New Jersey, USA) was regenerated with nickel sulfate following manufacturer's recommendations and equilibrated with 10 mM Tris-HCl, 20 mM NaCl; pH = 8.0. Previous disruption supernatant, containing Vip3Aa1, was applied to a XK-16 chromatographic column (Amersham-Biosciences, Uppsala, Sweden) loaded with 2 mL of IMAC matrix and operated at 30 cm h^{-1}. The adsorbed protein was eluted increasing imidazole concentration (100, 250 and 500 mM). A subsequent purification experiment (from 5 L-fermenter) was done applying the disruption supernatant containing Vip3Aa1 in 10 mM Tris-HCl, 20 mM NaCl, 100 mM imidazole; pH = 8.0. Next, elution fraction buffer was exchanged to 150 mM Phosphate Buffered Saline solution (PBS); pH = 8.0 by size-exclusion chromatography using Sephadex G-25 (Amersham-Biosciences, Uppsala, Sweden) operated following manufacture recommendations.

Vip3Aa1 characterization

Vip3Aa1 purity analysis by SDS-PAGE: Vip3Aa1 purity was measured by SDS-PAGE at 10% following method described by Laemmli (1970), combined by a densitometry analysis. All purified fractions were previously concentrated in a speed-vac concentrator (Speed-vac Plus® SC 110A, Model-RH6411, Instrument Inc. Farmingdale, NY, Canada) for 2 h.

Vip3Aa1 identity analysis by western-blotting: SDS-PAGE (10%) separated samples were transferred onto a Hybond-C extra membrane using a blotting system as it was reported by Towbin *et al*. (1979). A previously Vip3Aa1 preparation, also purified from *Escherichia coli*, was used as positive control.

Vip3Aa1 identity analysis by mass spectrometry: Sample containing Vip3Aa1 was applied in a SDS-PAGE (10%). Gel band visualized with Coomassie blue was washed with 1 mL of Milli-Q water for 5 min and with 500 μL of 250 mM NH_4HCO_3 solution in 50% acetonitrile, until discoloration.

Band was washed again with 1 mL of water for 3 min and next 90% acetonitrile was added until complete dehydration. Acetonitrile was removed and cubes were dried in a centrifuge (Savant, USA). Gel cubes were hydrated with 20 μL of 50 mM NH_4HCO_3 solution containing trypsin (Promega, Madison, USA) at 12.5 ng mL^{-1}. Peptides were extracted by micro-C18 ZipTip columns to be analyzed by a QTof-1 hybrid type orthogonal tandem mass spectrometer (Micromass, UK) (Gonzalez *et al*., 2003).

Vip3a1 and total protein concentration determination: Vip3Aa1 and total protein concentration determination was done following method described by (Lowry *et al*., 1951).

Vip3Aa1 insecticidal activity estimated by a bioassay using *Spodoptora frugiperda*: Liquid artificial diet components (5 g agar, 375 mL H_2O, 1.3 mL formaldehyde, 1.3 g ascorbic acid, 0.75 g sorbic acid, 1.1 g methyl paraben, 2.2 g vitamins, 1 g folic acid, 27.5 g beams, 22 g wheat germ, 11.5 g soybean meal, 8 g hydrolyzed of casein, 14 g yeast extract g, 1g salts) were mixed at 65°C. Then, purified Vip3Aa1 was applied at (2, 1, 0.5 and 0.25 μg mL^{-1}) of diet. Next, one second instar *Spodoptera frugiperda* larva was placed per well in 32 wells-plates and incubated in a WiseCube® WGC programmable growth chamber WGC-450 (DAHIAN Scientific Co. Ltd, Seul, Korea) at 28°C, 70% of relative humidity and a photo-period of 14 h:10 h (dark: light) for seven days. Diet supplemented with Bovine Serum Albumin (BSA) or without additional supplements was used as negative controls and the number of independent measurements was two.

Vip3Aa1 specific-polyclonal rabbit antibody generation: The purified Vip3Aa1 protein was used to generate polyclonal antibodies anti-Vip3Aa1 (α-Vip3Aa1) in rabbits provided by the National Center for Laboratory Animal Breeding, Cuba. Animals were immunized with 100 μg of Vip3Aa1 mixed with Freund's complete adjuvant (1:1 v/v), intramuscularly. Remaining two immunizations were carried out every 15 days but with Freund's incomplete adjuvant. Harvested blood samples were centrifuged for 15 min at 2900 g for isolating serum for polyclonal antibody preparation titration.

Vip3Aa1 specific polyclonal rabbit antibody serum titration by enzyme-linked immunosorbent assay (ELISA): A polystyrene 96-well microtiter plate (Maxisorp, Nunc, USA) was coated for 1 h at 37°C, with 10 μg mL^{-1} of Vip3Aa1 dissolved in carbonate/bicarbonate buffer; pH = 9.6 (100 μL $well^{-1}$). Plate was blocked with BSA (3%) for 1 h at 37°C and washed four times with 150 mM PBS/0.05% Tween 20; pH = 7.2. Then, serum was diluted in 150 mM PBS; pH = 7.6 and added to the plate to be incubated for 1 h at 37°C. A further plate wash step was performed again and 100 μL of an anti-rabbit antibodies conjugated with alkaline-phosphatase (SIGMA, St. Lois, Missouri, USA) diluted in 150 mM PBS; pH = 7.6 was applied to the plate and incubated for 1 h at 37°C. Finally, plate was incubated with p-nitrophenyl phosphate in 100 mM diethanolamine; pH = 9.8 at 37°C and absorbance was measured at 405 nm using an ELISA reader (Labsystems, Helsinki, Finland).

Vip3Aa1 specific polyclonal rabbit antibody quantification: The determination of Vip3Aa1 specific polyclonal rabbit antibody concentration was done at 280 nm and by Lowry method (Lowry *et al*., 1951).

Vip3Aa1 specific-polyclonal rabbit antibody purification: Polyclonal rabbit antibodies were purified by affinity chromatography using Protein A-Sepharose (Amersham-Biosciences, Uppsala, Sweden). The α-Vip3Aa1 serum was applied to a XK-26 chromatographic column (Amersham-Biosciences, Uppsala, Sweden) equilibrated with 150 mM PBS; pH = 8.0. Antibodies were eluted using 100 mM citric acid; pH = 3.0. The elution fraction pH was neutralized with 2 M Tris. Subsequently, a buffer exchange to 20 mM Tris/150 mM NaCl; pH = 7.6 was performed by size-exclusion chromatography with Sephadex G-25 (Amersham-Biosciences, Uppsala, Sweden) operate at 130 cm h^{-1}.

Corn plant cultivation: Corn plants genetically-transformed to express Vip3Aa1 (MIR162) and Cry1Fa1 (FR-Bt1); and non-transformed corn plants were grown under controlled conditions according to standard agriculture production practices of the Center for Genetic Engineering and Biotechnology of Havana, Cuba. Seeds were manually sown at 2 seeds per m^2 and harvested after three months of plant cultivation to be dried at 42±3°C for 3 days, store at 4°C and ground.

Soluble protein extraction from corn plant seeds: Forty grams of corn plant seeds were ground in a mill CEMOTEC 1090 (Sweden). Powder was suspended in 150 mM PBS/0.1% Tween 20; pH = 7.0 to be centrifuged at 6700 g, 4°C for 10 min. Supernatant was used to detect Vip3Aa1 by Dot-blot and for the biological assay performed to estimate Vip3Aa1 insecticide capacity against *Spodoptera frugiperda*.

Vip3Aa1 detection in genetically-modified corn plant seeds by dot-blot: The activation of a nitrocellulose membrane in methanol for 20 min was previously done. Then, it was placed in a Dot-blot equipment and 100 μL of each serial twofold diluted sample of ground seeds were applied. After that, it was blocked with 5% milk/150 mM PBS/0.05% Tween 20; pH = 7.6 for 1 h. Vip3Aa1 was detected with α-Vip3Aa1 polyclonal rabbit antibodies and a goat polyclonal antibody preparation, directed against rabbit immunoglobulins, conjugated with alkaline-phosphatase (SIGMA, St. Lois, Missouri, USA). The conjugate was incubated for 2 h at 37°C. Color development was visualized using 100 mM Tris-HCl/100 mM NaCl/5 mM MgCl2/150 μg mL^{-1} of nitro blue tetrazolium and 75 μg mL^{-1} of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega, Madison, USA).

Soluble protein extraction from genetically-modified corn plant leaves: Samples of leaves (0.03 g) were extracted with liquid nitrogen employed a polytron (IKA-WERK RW-20, Laboratory Equipment, USA). The extracted material was mixed with 150 mM PBS/0.1% Tween 20; pH = 7.0. Protein preparation was centrifuged at 6700 g for 10 min at 4°C and supernatant was then used to detect Vip3Aa1 by Western-blot and ELISA.

Vip3Aa1 detection in genetically-modified corn plant leaf extract by western-blot: The detection was performed using the same procedure employed in the section Vip3Aa1 identity analysis by Western-blotting.

Vip3Aa1 detection in genetically-modified corn plant leaf extract by ELISA: A 96-well microplate (Maxisorp, Nunc) was coated with 10 μg mL^{-1} of α-Vip3Aa1 polyclonal rabbit antibodies for 1 h at 37°C. Plate was washed 4 times with 150 mM PBS/0.05% Tween 20; pH = 7.6 and blocked with 3% BSA/150 mM PBS/0.05% Tween 20; pH = 7.6 for 1 h at 37°C. After washing step, samples of leaf macerated were applied in serial twofold dilutions (30, 15, 7.5, 3.75, 1.87 ng mL^{-1}) and incubated under the same conditions. Next, the plate was washed again and 100 μL of α-Vip3Aa1 polyclonal rabbit antibodies conjugated to horseradish peroxidase (SIGMA, St. Lois, Missouri, USA) was added and incubated for 1h at 37°C. After another washing step, 100 μL per well of peroxidase substrate solution was added. Reaction was stopped by adding 50 μL of 2 M H_2SO_4 and absorbance was measured at 492 nm using a microELISA reader (Labsystems, Helsinki, Finland).

Bioassays performed for measuring Vip3Aa1 insecticidal activity in genetically-modified corn plants: Liquid artificial diet components were prepared as it was mentioned above. On the other hand, the seed macerated supernatant was diluted from 1 g to 0.25 g in 150 mM PBS/0.1% Tween 20; pH = 7.0. Next, each dilution (1, 0.5 and 0.25 g) was mixed with 20 mL of diet and dispensed at 1 mL per well. Next, one larvae of *Spodoptera frugiperda* second install was placed per well in a 32 well-plate and incubated under the same condition mentioned above. Ground seeds of corn plants genetically-transformed to express Cry1Fa1 were used as positive control, whereas seed macerated of non-transgenic corn plants and a sample without any extra supplements were used as negative controls, respectively.

Statistical analysis: An ANOVA and a Multiple Range test were performed to compare study results carried out to measure Vip3Aa1 insecticidal capacity using 95% of statistical confidence. Medium Lethal Concentration (LC_{50}) of Vip3Aa1 protein was estimated by the R software (Crawley, 2005).

RESULTS

Gene encoding histidine tail-Vip3Aa1 toxin obtained from the AB88 Bt strain was cloned into the pQE30 expression vector to transform the JM-109 strain of *Escherichia coli* in this study. In this sense, Fig. 1 illustrates plasmid map used to transform JM-109 strain, where Vip3Aa1 expression regulation by PT5:T5 promoter/lac operator elements can be recognized while ampicillin was used as selection marker. It also shows an agarose electrophoresis picture of six transformed clones, where bands detected at 2.2 and 3.4 kb coresponded with Vip3Aa1 gene and pQE30 plasmid, respectively. From these transformed clones, clone number 1 (Lane 2) was selected to perform the rest of the study.

In consequence, Vip3Aa1 was expressed as a soluble protein and the yield measured in 300 mL-Erlenmeyer flasks and 5L-fermenter was 25.0 and 33.6 μg mL^{-1} of supernatants, respectively (Table 1). The Vip3Aa1 recovery of the disruption process, after two disruption cycles, was 82.6% (300 mL- Erlenmeyer flasks) and 80.0% (5 L-fermenter). The disruption step average recovery considering both processes was 81.3±1.8% (Table 1). Samples of both disruption processes were solubilized in 50 mL which corresponded with the minimum working volume of the French press employed and thus a maximum concentration factor of 100 times. Therefore the concentration factor could even be higher if another press could be used. Nevertheless, it is something than cannot be easily assumed by the influence of protein preparations highly concentrated on the efficiency of subsequent purification step.

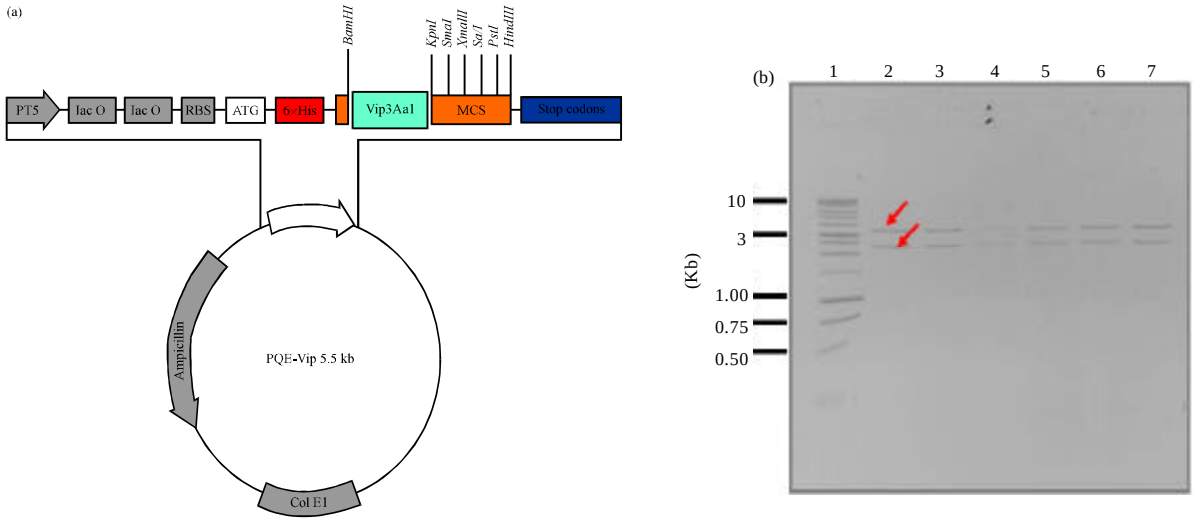


Fig. 1(a-b): (a) Plasmid map used to transform the JM-109 *E. coli* strain and (b) Agarose electrophoresis picture, bands observed at 2.2 and 3.4 kb coresponded with the Vip3Aa1 gen and pQE30 plasmid, respectively. Lane 1: Molecular Weight Marker 1 kb Leader (Promega, USA) and Lane 2-6: DNA of six different tranformed *E. coli* strains

Table 1: Summary of rabbit polyclonal antibody generation and Vip3Aa1 purification method results

Parameters	Title vs Vip3Aa1 (dil.)	Total protein amount (mg)	Vip3Aa1 amount (mg)	Vip3Aa1 recovery (%)	Concentration factor (times)	SDS-PAGE purity (%)	Vip3Aa1 yield (μg mL^{-1})
Fermentation supernatant (300 mL)	-	-	7.5	-	-	-	25.0
Disruption and centrifugation	-	-	6.2	82.6	6.00	-	-
Affinity chromatography application	-	-	6.2	-	-	-	-
Affinity chromatography non-bound	-	-	0.5	-	-	-	-
Affinity chromatography elution	-	-	4.2	67.7	12.00	-	-
Size-exclusion chromatography	-	-	4.0	95.3	24.00	99.2	-
Total	-	-	-	53.3	36.60	-	13.0
Fermentation supernatant (5000 mL)	-	-	167.8	-	-	-	33.6
Disruption and centrifugation	-	-	134.3	80.0	100.00	-	-
Affinity chromatography application	-	-	134.3	-	-	-	-
Affinity chromatography non-bound	-	-	38.5	-	-	-	-
Affinity chromatography elution	-	-	101.2	75.3	125.00	-	-
Size-exclusion chromatography	-	-	80.0	79.0	62.50	95.3	-
Total	-	-	-	47.6	62.50	-	16.0
Animal serum (20 mL)	1:30000±4142	-	-	-	-	-	-
Affinity chromatography elution	-	35	-	-	-	98.5	-
Size-exclusion chromatography	-	33	-	-	0.66	99.0	1650.0

The concentration factor was calculated with respect to the volume of sera or fermentation supernatant

Results of Vip3Aa1 affinity chromatography method revealed 71.5±5.3% (67.7%, 300 mL-Erlenmeyer flask and 75.3%, 5 L-fermenter) of recovery (Table 1). In this regards, first purification experiments performed with supernatant obtained from 300-mL-Erlenmeyer flask cultivation was done using a discontinuous gradient of imidazole (100, 250 and 500 mM). It allowed demonstrating that huge majority of Vip3Aa1 eluted from affinity chromatography column from 250 mM of imidazole. Nevertheless, fractions with non-representative amount of Vip3Aa1 were also detected in buffer containing 100 and 500 mM of imidazole, respectively. Next, to optimize this chromatography procedure, supernatant containing Vip3Aa1, produced from 5 L-fermenter was applied to the column with 100 mM of imidazole. It allowed to reduce chromatography time and to increase Vip3Aa1 recovery in almost 8%. However, Fig. 2b-c also evidenced that Vip3Aa1 was also detected in the non-bound fraction.

The detailed analysis of Vip3Aa1 recovery by each purification process evidenced following results; cell disruption+centrifugation (82.6%, 300 mL-Erlenmeyer and 80.0%, 5 L-fermenter), affinity chromatography (67.7%, 300 mL-Erlenmeyer and 75.3%, 5 L-fermenter) and size-exclusion chromatography (95.3%, 300 mL- Erlenmeyer and 79.0%, 5 L-fermenter). Purification

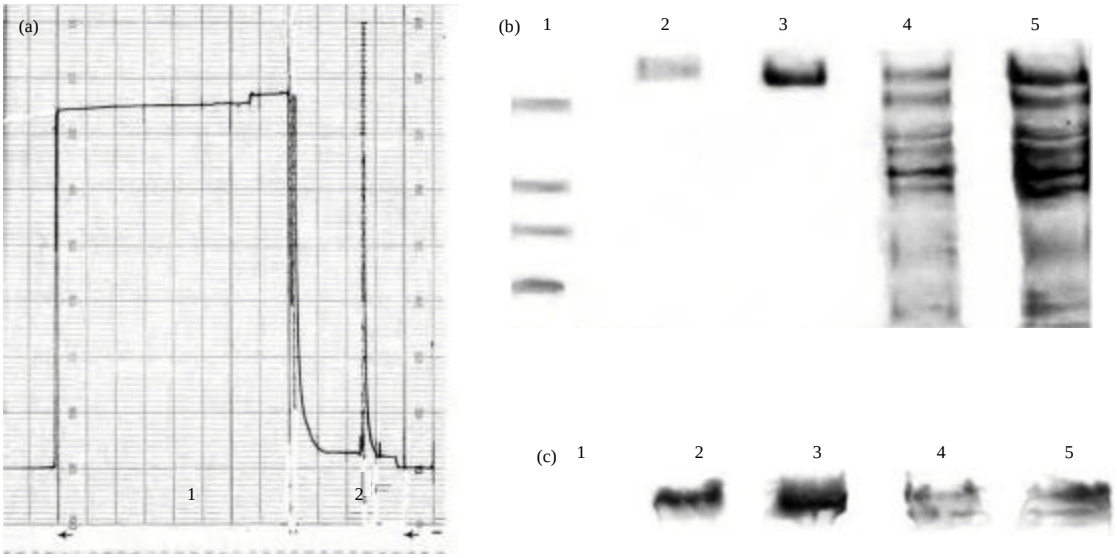


Fig. 2(a-c): (a) Metal affinity chromatography chromatogram observed in the Vip3Aa1 purification. 1, Non-bound fraction. 2, Elution fraction using Imidazole at 250 mM, (b) SDS-PAGE, Lane 1: Molecular Weight Marker: Bovine serum albumin (66 kDa), Chicken ovolabimun (45 kDa); Glyceraldyhe-3 phosphate (36 kDa) Bovine carbonic anhydrase (29 kDa), Lane 2: Recombinant Vip3Aa1 used as positive control, Lane 3: Elution fraction at 250 mM of Imidazole, Lane 4: Non-bound fraction. Lane 5, Disruption supernatant and (c) Western-blot performed to identify the Vip3Aa1. Lane 1: Molecular weight marker: Bovine serum albumin (66 kDa), Chicken ovolabimun (45 kDa); Glyceraldyhe-3 phosphate (36 kDa) Bovine carbonic anhydrase (29 kDa), Lane 2: Recombinant Vip3Aa1 used as positive control, Lane 3: Elution fraction at 250 mM of imidazole, Lane 4: Non-bound fraction and Lane 5: Disruption supernatant

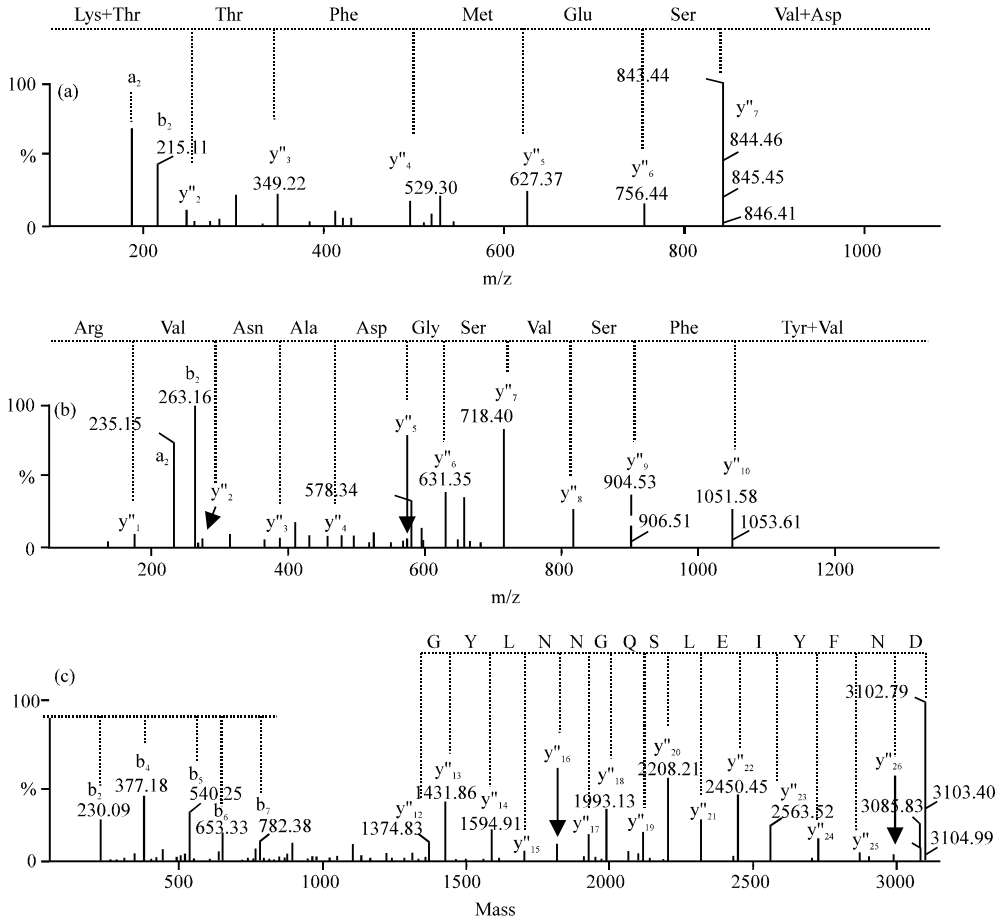
method total recovery ranged 53.3-47.6% for an average equivalent to 50.4±4.0% (Table 1). The maximum concentration factor of whole purification method observed was 62.5 times (Table 1). This data was estimated from the 5 L-fermenter process. Obviously, the step with higher contribution to concentration factor was the cell disruption step.

Concerning target protein characterization, the purity of purified Vip3Aa1 measured by SDS-PAGE and a densitometry analysis was 99.2% (300 mL-Erlenmeyer) and 95.2% (5 L-fermenter), allowing and an average 97.2±2.8% (Table 1, Fig. 2). Results of Vip3Aa1 characterization by mass spectrometry allowed verifying the expected total correspondence between DNA and amino acid sequences. In general, 789 amino acids were identified and no amino acid additions, deletions or substitutions were detected (Fig. 3). Summarizing, Vip3Aa1 purity and identity was considered very high to be used for specific antibody generation.

Before proceeding to polyclonal rabbit antibody generation, a biological assay was carried out to demonstrate recombinant Vip3Aa1 protein insecticidal capacity. In this sense, the bioassay showed pronounced differences in death rate values. At seventh day of experiments, most live larvae were arrested in their development and died. The death rate values obtained were 93.5±8.8% (2 μg mL^{-1}), 68.7±8.8% (1 μg mL^{-1}), 62.5% (0.5 μg mL^{-1}) and 68.7±8.8% (0.25 μg mL^{-1}). As it shown, statistical differences were detected among results of 2 μg mL^{-1} and results of the rest Vip3Aa1 assessed concentrations ($p = 0.0001$) (Table 2). The LC50 of Vip3Aa1 estimated by the R software was 0.346 μg mL^{-1} (fudicial limits: 0.246-0.485) of diet. Therefore, it corroborated that Vip3Aa1 expressed and purified from *Escherichia coli* retained its strong toxicity after being subjected to common biochemical steps used in protein purification.

In regards to results of polyclonal rabbit antibody generation, Vip3Aa1 in combination with Freund's complete-and incomplete-adjuvants showed a high immunogenicity capacity (1:30000±4142) in specific pathogen free 2 kg-body weight rabbits after a primary immunization and two re-immunization doses (Table 1).

Figure 4 shows Vip3Aa1quantification in seed macerated and extract of leaves of transgenic corn plants. The Dot-blot analysis showed 1 ng mL^{-1} as ViP3Aal



MNKN NT KLSTRALPSFIDYFNGIYGFATGIKDIMINMIFKTDTGGDLTLDEILKNQQLLNDISGKLDGVNGSLNDLIA
QGNLNT ELSK ELIKIANEQNQVLNDVNNKLDAINTMLRVYLPKITSMLDSVMKQNYALSLQIEYLSKQL QEISDKL
DII NVN VL INSTLEITPAYQRIKYVNEKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGF EFYLN
TFHDV MVGNNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAQAFLTLTTC RKLLGLADI DYTSIMN
E HLNKE KEEFRV NIL PTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALIGFEISNDSITVL KVYEKLKQN YQVD
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AEY RTS ANDDGV YMPLG VISE TFLTPINGFGLQADENSRLITLTCKSYLRELLLATD LSNKETK LIVPPSGF ISNIV
ENGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYTVKGKPSIHLKDENT
GYIHYEDTNNLEDYQTINKRFTTGTDLKGVYLILKSQNGDEAWGD NFIILE ISPSEK LLSPE LINTMNW TSTGSTN
ISGNTLTLYQGGRGILKQNLQLDSFSTYRVYFSVSGDANVRI RNSREV LFEKRYMSGAK DVSEMFTTK FEKDNFY
IELSQGNNLYGGPIVHFYD

Fig. 3(a-c): (a) Examples of ESI-MS/MS signals, m/z 529.24, (b) 657.36 and (c) 1034.87 corresponding with peptides ^{751}DVSEMFTTK^{759}, ^{721}VYFSVSGDANVR^{732}, ^{763}DNFYIELSQ GNNLYGGPIVHFYDVSIK^{789} of recombinant Vip3Aa1 and whole Vip3Aa1 amino acid sequence

detection limit and not reaction against seed macerated of transgenic corn plants transformed with Cry1Fa1 gene, non-genetically transformed corn plants and BSA used as negative controls (Fig. 4a). On the other hand, Fig. 4b-c illustrates results of Vip3Aa1 detection in extract of leaves of transgenic corn plants. As it shown, the Western-blot was also highly specific for Vip3Aa1, allowing detection of this protein without interference.

Figure 4d demonstrates the linear range of standard curve applied in the indirect sandwich ELISA designed to quantify Vip3Aa1. The linear relation between absorbance at 492 nm and Vip3Aa1 concentration ranged 30 ng mL^{-1}-2 μg mL^{-1}, regression coefficient was equal to 0.9920, detection limit (greater than blank absorbance+3xSD of blank absorbance value) was 1 ng mL^{-1} and quantification limit 30 ng mL^{-1} which is suitable for protein quantification.

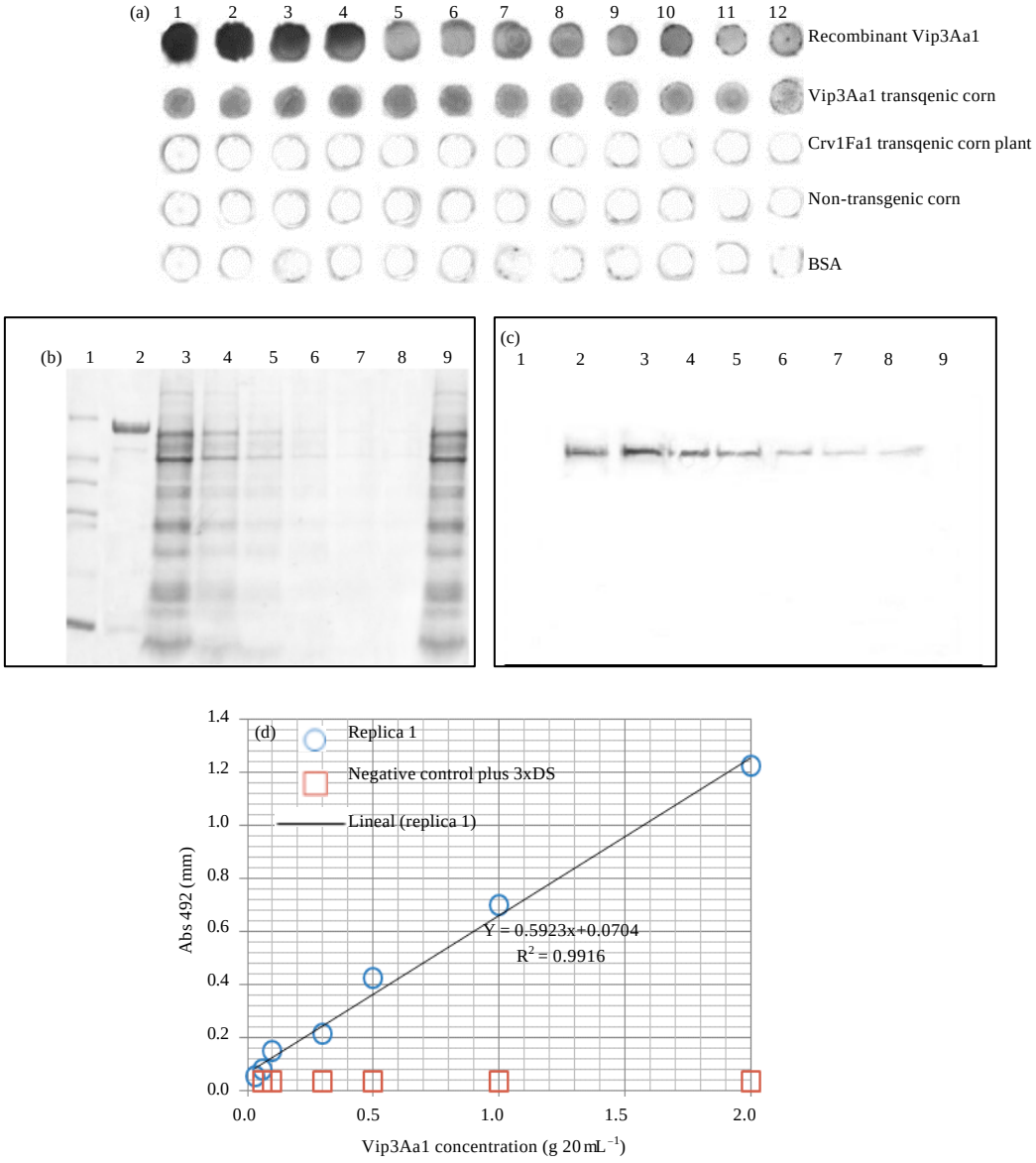

Fig. 4(a-d): Results of Vip3Aa1 detection in seeds and leaves of transgenic corn plants (a) Dot-blot done to detect Vip3Aa1 in seed macerated. Lane 1: Recombinant Vip3Aa1 used as standard curve (ranged 0.5 μg mL^{-1}-1.0 ng mL^{-1}) and positive control, Lane 2: Sample of transgenic corn plant seed macerated transformed with Cry1Fa1 gene (negative control), Lane 3: Macerated of corn plant seeds transformed with the Vip3Aa1 gene, Lane 4: Macerated of non-transformed corn plant seeds (negative control). In all cases (unless standard curve) the initial volume applied was 100 μL, (b) SDS-PAGE performed to measure Vip3Aa1 in extract of leaves of transgenic corn plants. Lane 1: Molecular weight marker, Lane 2: Recombinant Vip3Aa1 (positive control), Lane 3-8: Serial twofold dilutions of leaf extract samples of the Vip3Aa1 genetically transformed corn plants. Lane 9, recombinant Cry1Fa1, (c) Western-blot performed to measure Vip3Aa1 in extract of leaves of transgenic corn plants. Lane 1: Molecular weight marker, Lane 2: Recombinant Vip3Aa1 (positive control), Lane 3-8: Serial twofold dilutions of leaf extract samples of the Vip3Aa1 genetically transformed corn plants and Lane 9: recombinant Cry1Fa1 and (d) Vip3Aa 1standard curve used in the indirect ELISA showing a linear relation between Vip3Aa1 concentration and absorbance at 492 nm

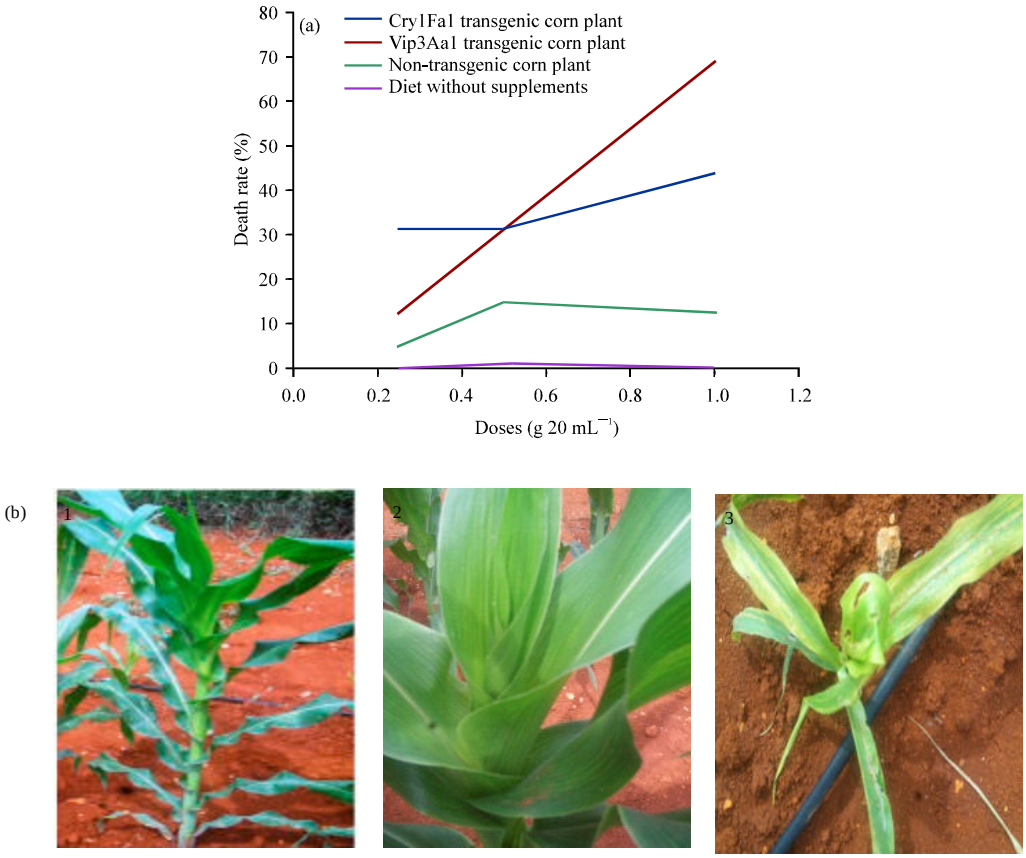

Fig. 5(a-b): Results of the biological assay performed to measure the mortality rate of *Spodoptera frugiperda* larvae fed with a diet supplemented with a seed macerated to corn plants transformed genetically to express the Vip3Aa1 (a) Illustration of mortality rate, (b) Pictures of corn plants cultivated during experiments in presence of corn moth (1) Cry1Fa1 transgenic corn plant, (2) Vip3Aa1 transgenic corn plant and (3) Non-transgenic plant

Table 2: Results of biological assay performed to measure purified recombinant Vip3Aa1 insecticidal capacity

Supplements added to the artificial diet	Control type	Doses (μg mL^{-1})	No. of larvae (Day 1)	No. of larvae (Day 7)	Death rate (%)	Multiple-tange test (α = 0.05)
Purified recombinant Vip3Aa1	-	2.00	8	0	100.0	X
Purified recombinant Vip3Aa1	-	2.00	8	1	87.5	X
Purified recombinant Vip3Aa1	-	1.00	8	2	75.0	X
Purified recombinant Vip3Aa1	-	1.00	8	3	62.5	X
Purified recombinant Vip3Aa1	-	0.50	8	3	62.5	X
Purified recombinant Vip3Aa1	-	0.50	8	3	62.5	X
Purified recombinant Vip3Aa1	-	0.25	8	3	62.5	X
Purified recombinant Vip3Aa1	-	0.25	8	2	75.0	X
Bovine serum albumin	Negative	2.00	8	8	0.0	X
Bovine serum albumin	Negative	2.00	8	8	0.0	X
Bovine serum albumin	Negative	1.00	8	8	0.0	X
Bovine serum albumin	Negative	1.00	8	8	0.0	X
Bovine serum albumin	Negative	0.50	8	8	0.0	X
Bovine serum albumin	Negative	0.50	8	8	0.0	X
Bovine serum albumin	Negative	0.25	8	8	0.0	X
Bovine serum albumin	Negative	0.25	8	8	0.0	X
Without supplements	Negative	-	8	8	0.0	X
Without supplements	Negative	-	8	8	0.0	X
ANOVA- p value	-	-	-	-	-	0.0001

Table 3: Results of the biological assay performed in *Spodoptera frugiperda*

Supplements to the artificial diet	Control types	Doses (g 20 mL^{-1})	No. of larvae (day 0)	No. of larvae (day 7)	Death rate (%)	Insecticidal factor vs controls (times)	Multiple-range test ($\alpha = 0.05$)
Vip3Aa1 transgenic corn seed macerated	-	1.0	16	5	68.7	-	X
Cry1Fa1 transgenic corn seed macerated	Positive	1.0	16	9	43.7	1.57	X
Non-transgenic corn seed macerated	Negative	1.0	16	14	12.5	5.49	X
Without supplements	Negative	1.0	16	16	0.0	-	X
Vip3Aa1 transgenic corn seed macerated	-	0.5	16	11	31.2	-	X
Cry1Fa1 transgenic corn seed macerated	Positive	0.5	16	11	31.2	-	X
Non-transgenic corn seed macerated	Negative	0.5	16	14	12.5	2.49	X
Without supplements	Negative	0.5	16	16	0.0	-	X
Vip3Aa1 transgenic corn seed macerated	-	0.25	16	14	7.0	-	X
Cry1Fa1 transgenic corn seed macerated	Positive	0.25	16	11	31.2	0.40	X
Non-transgenic corn seed macerated	Negative	0.25	16	16	5.0	2.50	X
Without supplements	Negative	0.25	16	16	0.0	-	X

Supplements were applied in the liquid artificial diets. Death rates were recorded on day 7 after the start of the assays

In this research, the biological screening performed to measure the insecticidal activity of Vip3Aa1 expressed in corn plants was also used to corroborate Vip3Aa1 detection in genetically-modified corn plants by the Dot-blot, Western-blot and ELISA. In this assay, the level of susceptibility of *Spodoptera frugiperda* larvae to Vip3Aa1 was measured based on the estimation of percentage of larvae that were able to survive with a diet supplemented with a seed macerated obtained from corn plants transformed genetically to express Vip3Aa1. Screening results demonstrated that Vip3Aa1 expressed in seeds of these corn plants was also highly insecticidal against *Spodoptera frugiperda* provoking an insect death rate as follow (68.7%, doses = 1 g 20 mL^{-1}; 31.2% doses = 0.5 g 20 mL^{-1} and 7.0%, 0.25 g 20 mL^{-1}) (Table 3, Fig. 5).

Plants expressing Vip3Aa1 showed and insecticidal factor of 1.57 and 5.49 times (at 1 g 20 mL^{-1}) in comparison with insecticidal factor exhibited by seed macerated of corn plants genetically-modified to express Cry1Fa1 and non-genetically modified corn plants, respectively. In the intermediate doses (0.5 g 20 mL^{-1}), difference in the insecticidal capacity was only observed against the seed macerated of non-genetically-transformed corn plants. Statistical analysis indicated the presence of significant differences among plants expressing Vip3Aa1 gene and non-genetically-modified plants (Table 3 and Fig. 5). Therefore, these results correlated well with results observed in the detection assays.

DISCUSSION

Insect-resistance crop widespread planting has raised a great concern over insect resistance development and non-transgenic plants cropping (Hofte and Whiteley, 1989; Bruns and Abel, 2003; Lee *et al*., 2003). Thus, this study aimed Vip3Aa1 production to be used for generating antibodies for further Vip3Aa1 identification in seeds and leaves of transgenic plants. The development of these assays was highly desired, because it would allow processing a large sample number during food screening process and for biosafety reasons; allowing the discrimination among genetically and non-genetically transformed plants in Cuba.

Vip3Aa1 expression and production: In this respect, *E. coli* which has been used as one of the most successful hosts to express heterologous proteins (Sorensen and Mortensen, 2005; Wu and Wong, 2005), was employed for expressing Vip3Aa1 protein. Several advantages justified the use of *E. coli* for such purposes. However, inclusion body formation frequently observed in *E. coli* as response to denatured protein accumulation was not underestimated. Because, as others researchers have reported, it could provoke low protein recovery and loss of biological activity of misfolded proteins during the target proteins refolding from inclusion bodies (Villaverde and Carrio, 2003).

In this sense, target protein and strain characteristics, cell culture medium, cultivation mode and mainly culture induction moment and temperature were taken into consideration to get Vip3Aa1 soluble expression. For instance, Vip3Aa1 is a protein with high solubility, usually expressed in the cell cytosol without the help of others proteins like chaperons and JM109 strain has JM107 recA1 strain genotype (Yamisch-Perron *et al*., 1985). Both properties have been quite effective to express heterologous proteins in a high level and in soluble manner. Though, authors emphasize that the high level of protein expression observed under this system (soluble protein+JM109 strain) could also be unexpectedly unfavorable for the expression of some proteins in soluble form.

In this respect, the heterologous protein over expression observed in this cultivation system is usually observed when it is operated in batch mode at high temperature (37°C). It produces a rapid bacterium growing reaching thus high densities. The use of high

temperatures could favor protein aggregation reaction due to the temperature dependence of hydrophobic interactions (Kiefhaber *et al*., 1991).

In this regard, other authors have demonstrated that an abrupt decrease in the cultivation temperature and vector promoter properties may affect target protein expression inhibiting replication, transcription and translation processes (Shaw and Ingraham, 1967; Vasina and Baneyx, 1996). For instance, temperature below 30°C and low induction levels result in higher amounts of soluble protein as consequence of cellular protein concentration reduction and a protein folding optimization (Weickert *et al*., 1996).

Considering these aspects, Vip3Aa1 expression method was designed to express this protein in a soluble form in this study. As results, Vip3Aa1 was expressed in a cytoplasmic soluble manner, Vip3Aa1 expression level estimated by SDS-PAGE was about 20% and maximum Vip3a1 yield was 33.6 μg mL^{-1} of supernatants (5 L-fermenter). Differences in the yield (1.34 times) observed between both cell culture systems (Erlenmeyer and fermenter) were expected due to fermenters allowed a better control of fermentation process. All these values were consistent with those reported by other authors (Wang *et al*., 2010).

Thereby, authors speculate that reason why this protein was expressed in this form, level and yield was a consequence of the induction method (IPTG) and low temperature (28°C) combination employed, because previous non-related to the subject of this report experiments performed using the same experimental conditions but a higher inductor concentration and temperature (37°C) in the induction moment revealed a higher Vip3Aa1 amount but almost all as inclusion bodies (data not shown).

In general, soluble protein purification is less expensive and time consuming than protein refolding and purification from inclusion bodies. Results of the disruption process performed here (French press), allowed obtaining a high recovery (>80%) of Vip3Aa1 in just two disruption cycles. According to other studies, results of this technique are more uniform and reach a complete disruption than those obtained with other methods such as mechanical or ultrasonic methods. However, contradictorily, other studies have reported a protein recovery over 90% when disruption conditions used in this study were applied but in just one disruption cycle (Buckland *et al*., 1976; Chisti and Moo-Young, 1986). Therefore, some optimization studies could be done to increase Vip3Aa1 recovery of disruption process and to reduce the disruption cycle number. The reduction of the disruption cycle number could be beneficial for reducing contaminant protein amount as well. Nevertheless, taking into consideration that next purification step is an affinity chromatography, it is unnecessary for the Vip3Aa1 purity and its use intention in this research.

The affinity of some fusion partners for immobilized ligands facilitates target protein purification. In consequence, the use of histidine tags added at the end of target protein amino acid sequence is one of the most successful examples implemented to improve purification system efficiency simplifying the whole downstream process (Hemdan and Porath, 1985; Gaberc-Porekar and Menart, 2005; Giraldo, 2007).

In this study, results of affinity chromatography method revealed that the huge majority of Vip3Aa1 eluted using 250 mM of imidazole and more than 70% of Vip3Aa1 recovery when two different elution procedures were applied. In addition, Vip3Aa1 was also detected in non-bound fractions and in buffer containing 100 and 500 mM of imidazole (non-representative amounts).

In contradiction to this result, other authors have reported almost 90% of recovery in the purification of recombinant histidine-tagged proteins such as green fluorescent protein when it was expressed in *E. coli* and purified by IMAC (Block *et al*., 2009; Dong *et al*., 2010). Perhaps, one of the reasons why Vip3Aa1 was observed in non-bound fractions was due the linear flow rate and packed bead height ratio used. Others explanations could be related with some degree of the ligand leakage after its interaction with Vip3Aa1. Though, this phenomenon is quite improbable in this type of matrix. Or, perhaps, the concentration of Vip3Aa1 used in the application material overcome steric hindrance of the binding of histidine-tagged Vip3Aa1 to the immobilized ligand as well as mass transport limitations of porous chromatographic media. Therefore, further experiments could also be done to improve the purification step recovery.

In regards to the whole purification process results, Vip3Aa1 recovery detailed analysis of each purification process step evidenced an average total recovery of 50.4±4.0% and 62.5 times as maximum concentration factor, in which the step with a higher contribution to the concentration factor was the cell disruption step. This total recovery is considered high for the application of this protein in this study but low with respect to the theoretical total purification process recovery (60-70%). The theoretical total purification process recovery was calculated by the multiplication of each theoretical purification step recovery (disruption-centrifugation (80%), IMAC (80-90%) and size-exclusion chromatography (95%) = 60.8-68.4%).

Vip3Aa1 characterization: The purified Vip3Aa1 average purity measured by SDS-PAGE and densitometry was 97.2±2.8%. This purity result was very high and

consistent with the fact the IAMC leads to high purity upon single-step chromatographic purification in many cases (Hahn *et al*., 2003; Queiroz *et al*., 2001). Differences observed in the Vip3Aa1 purity between 300 mL-Erlenmeyer and 5 L-fermenter experiments could be explained by the fact that coelution of non-specific proteins from this chromatography support was provoke by the high protein concentration obtained in the 5-L fermenter supernatant after the disruption step. Other authors have also demonstrated the copurification of proteins together with histidine-tagged proteins from *E. coli* supernatants. It can be done due to differences reasons such as the presence of proteins with natural metal-binding motifs, proteins with histidine clusters on their surfaces, proteins that bind to heterologously expressed histidine-tagged proteins and proteins with affinity to agarose-based supports (Bolanos-Garcia and Davies, 2006).

As rule, these impurities are stress-responsive proteins with affinity by metals, suggesting that cultivation conditions have influence on their amount and appearance as a contaminating species of the target protein preparation. Perhaps the presence of this kind of proteins was stimulated under the culture conditions used in the 5 L-fermenter used in this study. However, the purity degree of the Vip3Aa1 preparation was enough for the further identity analysis and insecticidal capacity demonstration.

As it was expected, Vip3Aa1 mass spectrometry analysis allowed verify correspondence between DNA and amino acid sequences. The total number of reported amino acids (789) was identified and no substitutions were detected. Therefore, it allowed ratifying *E. coli* properties to express heterologous proteins (Bingle *et al*., 2000; Murashima *et al*., 2002; Mergulhao *et al*., 2005).

The Vip3Aa1 insecticidal activity tested after purification process using *Spodoptera frugiperda* showed pronounced differences in death rate values when mortality was measured at seventh day of experiment. Values ranged in a protein doses dependent manner. The maximum death rate value was estimated at 2 μg mL^{-1} (93.5±8.8%) for a LC_{50} equal to 0.346 μg mL^{-1} of diet. Therefore, this experiment corroborated the high susceptibility of *Spodoptera frugiperda* to this protein and also that Vip3Aa1 expressed and purified from this *E. coli* strain retained its strong toxicity after being subjected to subsequent purification steps.

Rabbit polyclonal antibody generation: Once purified Vip3Aa1 properties were demonstrated; authors proceed then to raise polyclonal antibodies in rabbits to carry out Vip3Aa1 quantification from transgenic corn plants. In this sense, Vip3Aa1 capacity to raise polyclonal antibodies in rabbits was considered high, >1:30000 as dilution factor. This titer satisfices titer needed to purify antibodies from rabbit sera but was apparently lower than titers estimated by other researchers. In this study, only one primary immunization and two boosters were used with 100 μg of Vip3a1 per animal instead of a primary immunization and four boosters with a higher amount of Vip3Aa1. Possibly, differences observed in the capacity to raise antibodies in rabbits between these studies could be explained by the protein amount and immunization protocol used and not by differences in Vip3Aa1 properties. To corroborate it, final amount of polyclonal rabbit antibodies obtained from rabbit sera (20 mL) was 33 mg which means a yield equivalent to 1.65 mg of purified polyclonal antibody per milliliter of the initial serum volume. This result cannot easily be contrasted with the scientific literature because it is not usually reported.

Vip3Aa1 quantification in seeds and leaves of transgenic corn plants: Immunological assays have been broadly used for non-purified sample screening and specific quantification of target proteins expressed and purified from transgenic plants (Kumar *et al*., 2010). As it was mentioned earlier, all this previous purification and characterization work was done to standardize biological assays for the detection of events such as MIR162, allowing the discrimination among non-transgenic and transgenic corn plants, within other subjects.

Besides, due to the high homology (>80%) in the Vip3 family amino acid sequence, polyclonal antibody based immunoassays employed in this study could also be used to detect other events related with proteins belonging to this protein family. This is perhaps, the most remarkable advantage of polyclonal antibody use in comparison with monoclonal antibodies. Another important advantage of polyclonal antibody use is the capacity to detect Vip3Aa1 fragments in genetically-modified plants, reducing the chance to give negative results.

In this study, Dot-blot assay was used to detect Vip3Aa1 from corn seed macerated because quantification by ELISA of this protein in seed samples was characterized by a great interference. Nevertheless, this immunoassay demonstrated to be quite sensitive and specific. Summarizing, sensitivity observed of the developed Dot-blot assay was in the nanogram range which is enough to quantify any recombinant proteins expressed in seeds of transgenic plants and coincident to other reports (Fantozzi *et al*., 2007; Wang *et al*., 2007).

The Western-blot assay performed to detect Vip3Aa1 in corn leaf extract was also highly specific for Vip3Aa1. This analysis also allowed concluding that this protein molecule did not suffer degradation or aggregation in the extract of corn leaves. In addition, results observed in the application of ELISA for the quantification of Vip3Aa1 from corn leaf extracts were also coincident with other reports in which a quantification limit in the nanogram range have been suitable for recombinant protein quantification. In this sense, detection and quantification limits could be lower if monoclonal antibodies directed against Vip3Aa1 were used. Therefore, validation works of immunoassays using monoclonal antibodies, directed against the Vip3 family common amino acid sequence, to quantify Vip3Aa1 will be reported by this group in a further work.

Finally, as Vip3Aa1 has shown a high insecticidal capacity against *Spodoptora frugiperda,* a biological assay was carried out to corroborate results of Vip3Aa1 determinations by Dot-blot, Western-blot and ELISA. This protein is produced as a protoxin which is cut by proteases in the larvae midgut rendering an active toxin which may provoke an extensive disintegration of epithelial tissue and larvae death (Lee *et al*., 2003).

In this sense, morality rate estimated at the end of the *in vivo* experiments with *Spodoptora frugiperda* also demonstrated the presence of Vip3Aa1 in seed macerated of genetically-transformed corn plants detected by *in vitro* assays mentioned above. Therefore, immunoassays established in this study would allow the discrimination among transformed and non-transformed corn plants and processing of a large sample number. The technical value of these immunoassays is even higher if researchers consider that an emergent insect resistance to these insecticidal proteins has been postulated.

CONCLUSION

The Vip3Aa1 was expressed in soluble form in *Escherichia coli* JM-109 strain and purified by metal-chelate affinity chromatography with high purity, without modifications in amino acid sequence, high immunogenicity in rabbits and high insecticidal capacity against *Spodoptora frugiperda*. Immunoassays developed in this study can be successfully used to detect Vip3Aa1 in genetically-modified corn plants, allowing discrimination between modified-and non-modified-genetically corn seeds and plants to regulate this kind of genetically-modified plants in Cuba.

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