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Monoclonal Antibody Generation and Characterization for Vip3Aa20 Protein Quantification in Transgenic Corn Plants

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Abstract

Numerous standardized techniques for detection and quantification of proteins are based on polyclonal antibody (pAb) use. However, because pAbs are a heterogeneous mixture of antibodies, there is the possibility of non-specific interactions or cross-reactions with non-related proteins, which is a disadvantage in the detection and quantification of target proteins. Therefore, the main objective of this study was to generate and characterize monoclonal antibodies (mAbs) for quantifying the Vip3Aa20 protein of *Bacillus thuringiensis (Bt)* expressed in event MIR162 transgenic corn plants.

The Vip3Aa20 bioinsecticidal protein of *Bt* shares 99.7% of homology with the Vip3Aa1 protein, which has already been successfully isolated by affinity chromatography on metal chelate. For this reason, Vip3Aa1 was chosen as an antigen for obtaining mouse mAbs directed against these *Bt* δ-endotoxins. The mAbs were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/Western blot, high-pressure liquid chromatography gel filtration (HPLC-GF), enzyme-linked immunosorbent assay (ELISA), and association constant. In general, results showed that the use of the highly purified mAbs not only allowed the specific and accurate quantification of Vip3Aa20 in extracts of macerated leaves of event MIR162 transgenic corn plants, but also statistically discriminated ($p=0.000$) between homozygous (range: 180–360 ng/mL, mean ± standard deviation [SD]: 241.7 ± 43.2 ng/mL) and hemizygous (range: 0–140 ng/mL, mean ± SD: 51.7 ± 40.8 ng/mL) transgenic corn plants grown under the same experimental conditions.

Introduction

Bacillus thuringiensis (Bt) is a facultative aerobic bacterium that is Gram-positive, spore-forming, and motile.[1] It is ubiquitous in nature, populating the surfaces of different plant leaves and soils, and is recognized today as the world leader of biopesticides.[2] Among pathogenicity factors that characterize this bacterium, the most important are the parasporal inclusion bodies composed of the δ-endotoxins Cry and Cyt. The former is used the most in the control of insect pests due to the high selectivity demonstrated by some species.[3]

Cry endotoxins affect some varieties of insects belonging to the *Lepidoptera, Diptera, Homoptera, Coleoptera, Hymenoptera, Orthoptera, and Mallophaga* orders; and also some species of nematodes, mites, and protozoa.[4] This feature has been used extensively in the realm of bioinsecticides since the mid-20th century.[5] It is also reported that the Cry endotoxin is activated after ingestion, producing pores in the midgut brush border membrane of susceptible larvae, causing insect death through starvation and septicemia.[6] To date, hundreds of *Bt* δ-endotoxins have been identified[7] and the genes coding these *Bt* δ-endotoxins have been used for generating modified crops resistant to insects.[8] However, after continuous exposure to a particular *Bt* δ-endotoxin-modified transgenic corn, the possibility of pests becoming resistant exists, as referenced by James with the Cry1Fa endotoxin in event TC1507 transgenic corn.[8]

To better manage this risk, precisely combining the same transgenic plant insecticidal endotoxins with different mechanisms of action (MOA) could be one solution. By doing this, during the logarithmic growth phase of *Bt*, other insecticidal proteins called vegetative insecticidal proteins (Vips) are also produced.[9] For instance, the Vip1 and Vip2 groups of binary endotoxins are highly insecticidal to some pests of the *Coleoptera* and *Hemiptera* orders.[10] Included in the Vip3 group of endotoxins is the Vip3Aa1 protein, which was first in the group to be described as highly insecticidal to lepidopterans such as the armyworm, *Spodoptera frugiperda*.[11] Vip toxin MOAs have not been fully elucidated yet, but it has been observed that they kill insects

via epithelial cell lysis of the midgut by forming pores in cell membranes.[12,13] Furthermore, it was shown that Cry and Vip3A endotoxins recognize different receptors in the midgut of susceptible larvae, which supports the use of Vip3A toxins in controlling Cry-resistant pests.[14] Therefore, standardizing tools is crucial for detecting and quantifying Vip3Aa proteins in genetically modified corn plants.[15]

To address the issue, authors used polyclonal antibodies (pAbs) obtained from rabbits immunized with the Vip3Aa1 protein purified from *Escherichia coli* (*E. coli*) cells transformed with the plasmid pQE30-Vip3Aa1.[16] Unfortunately, unwanted interactions of rabbit pAbs with the extract of macerated leaves from event TC1507 transgenic corn plants and non-transgenic corn plants were observed. It affected the specificity of pAbs for Vip3Aa1, the quantification limit of the assay, and also the discrimination between homozygous and hemizygous transgenic plants.

The drawbacks mentioned above were taken into account when developing the study parameters discussed in this paper. We have focused on generating and characterizing monoclonal antibodies (mAbs) for identifying and quantifying Vip3Aa20 expressed in event MIR162-modified corn plants—without the interference of non-transgenic and transgenic event TC1507 plants. As a secondary study subject, the capacity of the mAb for discriminating between homozygous and hemizygous transgenic corn plants grown under the same experimental conditions was also analyzed.

Materials and Methods

Vip3Aa1 Production

The JM-109 strain of *E. coli* (Invitrogen/Thermo Fisher Scientific) containing the pQE30-Vip3Aa1 plasmid was thawed on ice for 15 minutes (min) and dispersed in a plate containing the solid Luria-Bertani medium (LB) consisting of 5 g/L yeast extract, 10 g/L tryptone, 10 g/L sodium chloride, 0.05 g/L ampicillin, and agar. After 15 hours (h) of incubation at 37°C, a colony was inoculated into an Erlenmeyer flask containing 125 mL of LB and incubated for 15 h at 37°C with vigorous stirring. Subsequently, it was used to inoculate a 5 L fermentor (BE Marubishi) containing LB. Vip3Aa1 expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the medium. This mixture was incubated at 28°C for 15 h. Next, the culture was centrifuged at 3300 ×*g* for 30 min at 4°C and the resulting pellet was solubilized in the lysis buffer consisting of 10 mM Tris-HCl, 20 mM sodium chloride, pH 8.0, with a cocktail of protease (Promega). Once solubilized, cells were disrupted by a French press in two cycles under pressures between 1000–1500 kPa. The mixture obtained was centrifuged at 31,000 ×*g* for 30 min at 4°C, and the supernatant was taken for Vip3Aa1 purification.[16]

Vip3Aa1 Purification

The Vip3Aa1 was purified using affinity chromatography (metal chelates). The IMAC matrix (GE Healthcare Life Sciences) was equilibrated with 10 mM Tris-HCl, 20 mM sodium chloride buffer, and 100 mM imidazole, pH 8.0. The supernatant was applied on an XK 16 column (GE Healthcare Life Sciences) loaded with 2 mL of matrix previously activated with nickel sulfate at a linear flow rate of 30 cm/h. The Vip3Aa1 protein was eluted with 250 mM imidazole dissolved in application buffer. The eluted fraction underwent buffer exchange with 150 mM phosphate buffered saline (PBS), at a pH of 8, by gel filtration chromatography using an XK 16 column packed with 60 mL of Sephadex G-25 (GE Healthcare Life Sciences). The linear flow rate used was 100 cm/h and the sample volume applied to the column was up to 30% of column volume.[16]

Immunogen Preparation

The immunogen used to generate the anti-Vip3Aa1 mAbs contained the previously purified Vip3Aa1 protein emulsified in Freund's complete adjuvant (Sigma-Aldrich). Freund's complete adjuvant was used in the immunization while in the remaining booster doses were done with Freund's incomplete adjuvant. The emulsified mixture was prepared in a total volume of 100 µL at a Vip3Aa1 concentration equal to 0.5 µg/µL.

Mouse Immunization

Five BALB/c mice of 24 ± 1 g were immunized and boosted four times with the immunogen using an interval of 14 days among doses of 100 µL at an amount of 50 µg per dose. At the end, the chosen animals were injected intraperitoneally with the immunogen 72 h before the splenectomy.

X63Ag8.653 Myeloma Cell Culture

X63Ag8.653 myeloma cells were thawed in a 37°C thermostatic bath and centrifuged for 10 min at 130 ×*g* to remove the freezing medium. Cells were resuspended in RPMI 1640 medium (Gibco/Thermo Fisher Scientific) supplemented with 8% (v/v) of fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), 2 mM L-glutamine (Gibco/Thermo Fisher Scientific), 1 mM sodium pyruvate (Sigma-Aldrich) and 17 mM sodium hydrogen carbonate (Merck KGaA). The cell viability was estimated by using trypan blue as vital dye.[17]

Hybridoma Generation

Hybridomas were obtained by the method described by Köhler and Milstein.[18] Lymphocytes extracted from the mouse spleen were fused in the presence of 1450 MW polyethylene glycol (PEG) solution (Sigma-Aldrich) 50% (v/v) to

the X63Ag8.653 myeloma at a rate of ten lymphocyte cells per one myeloma cell. For this purpose, eleven 96-well plates (Maxisorp NUNC/Thermo Fisher Scientific) were seeded in RPMI 1640 medium supplemented with 8% FBS and hypoxanthine-aminopterin-thymidine (HAT) (Sigma-Aldrich) and maintained at 37°C in an atmosphere of 5% CO_2. After a culture time of 192 h, the culture medium was replaced with HAT medium supplemented with hypoxanthine-thymidine (HT) (Sigma-Aldrich). The selection of positive wells was done by a specific enzyme-linked immunosorbent assay (ELISA) using rabbit pAb anti-mouse IgG preparation conjugated to horseradish peroxidase (Promega). Wells with the highest absorbance were selected for cloning by a limiting dilution method at a rate of one cell per well.

Hybridoma Culture for Mouse Inoculation

Cells were grown in 250 mL spinner flasks, from 3×10^5 cells/mL, in RPMI 1640 supplemented with 8% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 17 mM sodium hydrogen carbonate. Cells were maintained at 37°C in an atmosphere of 5–7% CO_2 and the medium was replaced every 48 h up to the maximum viable cell density (~1×10^6 cells/mL).

Ascites Production

Ten BALB/c mice weighing 24 ± 2 g were used for ascites production. Animals were maintained at 22°C, 50% humidity, keeping levels of ammonia low. Ten days before inoculation, each animal received 0.5 mL of liquid petrolatum intraperitoneally. In all cases, the amount of inoculated cells was 1.0–1.5 × 10^6 cells/mL. The inoculum volume was always 1 mL per animal. Next, ascites was harvested ten days post-cell inoculation and filtered (0.45–0.22 µm pore size). Finally, the filtered ascites was stored at –20°C until later mAb purification.

Purification of mAb Directed Against Vip3Aa1

Anti-Vip3Aa1 mAbs contained in the filtrated ascites were purified by affinity chromatography using Protein A Sepharose CL-4B Fast Flow (GE Healthcare Life Sciences). To this, 1.5 M glycine/3 M sodium chloride, pH 8.9, and 100 mM citric acid were used as adsorption and elution buffers, respectively. The mAbs were eluted by a pH gradient starting with a pH of 6.0 followed by 5.0, 4.0, and then 3.0. An XK 26/20 column (GE Healthcare Life Sciences) loaded with 7 mL of Protein A Sepharose and operated at a linear flow rate of 90 cm/h was used. Extensive washings were performed with 1.5 M glycine/3 M sodium chloride, pH 8.9, to remove contaminants. Next, a buffer exchange was performed by size-exclusion chromatography (SEC) using a matrix of 216 mL Sephadex G-25 coarse (GE Healthcare Life Sciences), 20 mM Tris/150 mM sodium chloride, pH 7.6, for the mobile phase, and an XK 50 column (GE Healthcare Life Sciences). The linear flow rate used was 19 cm/h. Finally, mAbs were concentrated by a 10 kDa membrane and filtered by 0.22 µm under sterile conditions.

Total Protein Concentration Determination

The total protein concentration was determined by the method described by Lowry *et al*.[19]

Vip3Aa1 and Anti-Vip3Aa1 mAb Purity Analysis

The purity of Vip3Aa1 and anti-Vip3Aa1 mAbs were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 10 and 12% respectively, following the method described by Laemmli[20] combined with a densitometry analysis. The amount of protein applied was always 20 µg.

Determination of mAb Molecular Homogeneity

The molecular homogeneity of mAb was determined based on the relationship between the molecular weight and the retention time in a high-pressure liquid chromatography gel filtration (HPLC-GF). The system used included: a Shimadzu HPLC system with a wavelength of 226 nm, TSKgel G3000 SWxl column (TosoHaas) and BioCrom data processing program (Center for Genetic Engineering and Biotechnology [CIGB]), 10 mM sodium phosphate/150 mM sodium chloride, pH 7.0, was used as mobile phase at a flow rate of 0.2 mL/min and a sample volume of 100 µL.

Characterization of the Epitope Recognized by mAb Specific for Vip3Aa1

In order to demonstrate if epitopes recognized by mAbs specific for Vip3Aa1 are continuous or discontinuous, Vip3Aa1 protein was applied to a SDS-PAGE (10%) as described above. The gel was then transferred to a Hybond-C membrane (GE Healthcare Life Sciences) using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The membrane was incubated with mAbcigbVip3Aa1-1, mAbcigbVip3Aa1-2, and mAbcigbVip3Aa1-3 separately, as suggested by Towbin *et al*.[21] After 1 h of incubation at room temperature followed by a membrane wash with 150 mM PBS/0.05% Tween 20, pH 7.0, a preparation containing the rabbit pAb anti-mouse IgG conjugated to horseradish peroxidase was applied to the plate and incubated again under the same conditions. For developing the reaction, a mixture of 150 mM PBS, 10 mg/mL 1,2′-diaminobenzidine (Sigma-Aldrich), and hydrogen peroxide 30% (Merck KGaA) was prepared in a total volume of 12 mL. The reaction was stopped by adding water to the membrane. Bovine serum albumin (BSA) was used as negative control.

Determination of mAb Subclass

The 96-well plates were coated with 10 µg/mL and 5 µg/mL of Vip3Aa1 protein using a buffer of 100 mM sodium carbonate/100 mM sodium bicarbonate, pH 9.6, and incubated at 37°C for 1 h. Then, several washes with 150 mM PBS/0.05% Tween 20, pH 7.0, were done and the plates were blocked with 3% BSA. After 1 h of incubation at 37°C, mAbs directed against Vip3Aa1 were added to plates and incubated again under the same conditions. Next, 100 µL of sheep pAb anti-mouse IgG1 and IgG2a

conjugated to horseradish peroxidase (Sigma-Aldrich) diluted in 150 mM PBS/0.05% Tween 20, pH 7.6, were added separately. The reaction was developed with 100 µL of a mixture containing the substrate buffer for peroxidase, 30% hydrogen peroxide, and *o*-Phenylenediamine (Sigma-Aldrich). The reaction was stopped with 50 µL of 2 M sulfuric acid and the absorbance was measured at 492 nm in an ELISA microplate reader (Labsystems).

Determination of Association Constant of mAbs Directed Against Vip3Aa1

The 96-well plates were coated with two concentrations of Vip3Aa1 protein (1.25 and 0.625 µg/mL) and incubated at 37°C for 1 h. Next, the respective mAb samples were applied and incubated at 37°C for 1 h (in serial dilutions of 781 ng/mL for mAbcigbVip3Aa1-1 and 312 ng/mL for both mAbcigbVip3Aa1-2 and mAbcigbVip3Aa1-3). Afterward, plates were washed with 150 mM PBS/0.05% Tween 20, pH 7.0. Sheep pAb anti-mouse IgG1 conjugated to horseradish peroxidase diluted in 150 mM PBS/0.05% Tween 20, pH 7.0, was applied for 1 h at 37°C. The plates were washed and the substrate solution for horseradish peroxidase was added. The reaction was stopped with 50 µL/well of 2 M sulfuric acid, and the absorbance was measured at 492 nm in the microplate reader. The mAb association constant was estimated by the method described by Beatty *et al*.[22]

Conjugation of mAbcigbVip3Aa1-2 to Horseradish Peroxidase

Horseradish peroxidase (2 mg/mL) and 5 mg/mL of mAbcigbVip3Aa1-2 were used along with a sodium periodate activator solution (200 µL) (Sigma-Aldrich). The stirring process was performed in 4°C room and protected from light exposure. The mixture was stabilized with 4 mg/mL of sodium borohydride (Sigma-Aldrich) for 2 h. The conjugate was then purified by SEC using Sephacryl SR-200 as matrix (GE Healthcare Life Sciences). The absorbance of collected fractions was measured at 405 and 280 nm and the activity of the antibody conjugate was estimated using the absorbance formula RZ = OD (405 nm)/OD (280 nm). The conjugate was preserved by adding glycerol (v/v) at –20°C.

Sandwich ELISA for Determining the Antibody Used as Vip3Aa1 Capture

A 96-well plate was divided into two sections and coated with 10 µg/mL of mAbcigbVip3Aa1-1 in one section and 10 µg/mL mAbcigbVip3Aa1-3 in the other section. The plate was incubated for 1 h at 37°C. Following incubation, the plate was washed with 150 mM PBS/0.05% Tween 20, pH 7.0, and blocked with 3% BSA. Then double serial dilutions of the Vip3Aa1 protein (starting from 1 µg/µL) were applied. After incubation for 1 h at 37°C, the mAbcigbVip3Aa1-2 conjugated with horseradish peroxidase was added and incubated again under the same conditions. Subsequently, the horseradish peroxidase substrate buffer was also added to the plate for 15 min. As before, the reaction was stopped with 50 µL of 2 M sulfuric acid and the absorbance was measured at 492 nm in the ELISA microplate reader.

Extraction of Soluble Proteins From Transgenic Corn Plant Leaves

Three different types of corn plant leaves were collected in quantities of 0.03 g: (a) event MIR162 transgenic plants; (b) event TC1507 transgenic plants; and (c) non-transgenic plants, which were independently macerated in liquid nitrogen using an RW 20 polytron (IKA). The macerated materials were each mixed with 150 mM PBS/0.1% Tween 20, pH 7.0, and centrifuged at 6700 ×*g* at 4°C for 10 min. The supernatants were used to detect Vip3Aa20 by the ELISA method described below.

Copy Number Determination of Gene-Coding Vip3Aa20 Protein in Event MIR162 Transgenic Plants

The extraction of the genomic DNA from transgenic and non-transgenic corn plants was done according to the protocol described by Shure *et al*.[23] The concentration of each sample was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and adjusted to 50 ng/µL. The real-time polymerase chain reaction (PCR) was performed using the Joint Research Centre protocol[24] described by Shure *et al*.[23] The event MIR162 detection system incorporated the QuantiTect SYBR Green PCR reagent kit (Qiagen) and Rotor-Gene 3000 thermal cycler (Corbett Research/Qiagen). The Δ cycle threshold (Ct) value for each sample was determined and used to estimate the number of gene copies coding Vip3Aa20 protein following the methodology used by Weng *et al*.[25]

Vip3Aa20 Quantification in Transgenic Event MIR162 Corn Plant Leaves

A 96-well plate was coated with 10 µg/mL of mAbcigbVip3Aa1-1 using a buffer of 100 mM sodium carbonate/100 mM sodium bicarbonate, pH 9.6. After 45 min of incubation at 37°C, the plate was washed four times with 150 mM PBS/0.1% Tween 20, pH 7.0, and blocked with 3% BSA. The concentrations of Vip3Aa1 standard curve were used (800, 400, 200, 100, 50, 25, and 12.5 ng/mL). The same incubation and washing methods were used for the mAbcigbVip3Aa1-2 conjugation. The reaction was developed with an enzyme substrate containing 30% hydrogen peroxide and *o*-Phenylenediamine. The reaction was stopped with 50 µL of 2 M sulfuric acid, and the absorbance was measured at 492 nm in the ELISA microplate reader. Event TC1507 plants and non-transgenic plants were used as negative controls.

Statistical Analysis

An analysis of variance (ANOVA) test was done to compare the concentration of Vip3Aa20 in event MIR162 transgenic plants, event TC1507 transgenic plants, and non-transgenic plants. The Duncan's multiple range test was done to discriminate among group of plants with statistical differences. The confidence limit used was 0.05.

Results and Discussion

It is essential that genetically modified crops be monitored for introgression. Numerous protein and nucleic acid detection and quantification techniques have been developed to assess consistent recombinant expression of insecticidal endotoxins bred into the seed stock.[26] These techniques are effective, relatively fast, and allow for the processing of large numbers of samples with high precision and specificity to easily discriminate between transgenic and non-transgenic plants.[27] These techniques are also very important for controlling the quality of seeds and plants, and for assessing environmental risks. However, in the case of immunoassays for *Bt* endotoxin detection and quantification, some of them have been developed on the basis of using pAbs directed against the Vip3Aa1 endotoxin.[28] In a previous study[16], some rabbit pAb interactions with the extract of macerated leaves of event TC1507 transgenic corn plants and non-transgenic corn plants were detected, affecting the specificity for Vip proteins, the quantification limits of the assay, and also the discrimination among transgenic plants. Therefore, this study was designed to characterize mAbs for identifying and quantifying Vip3Aa20 expressed in event MIR162 transgenic plants, without interference from event TC1507 transgenic plants and non-transgenic plants, and also to discriminate between homozygous and hemizygous transgenic corn plants grown under the same experimental conditions.

To reach these two goals, the Vip3Aa1 protein purified by affinity chromatography with metal chelates, and characterized as described by Hernández *et al*.[16], was used to immunize five BALB/c mice. **Table 1** shows the hyperimmune serum titers for each mouse. The extraction of blood and serum titers for all mice was performed and measured ten days after immunization (estimated titer range 1:2048–1:4096) and three days after the third booster or reactivation (estimated titer range 1:64,000–1:128,000). Using this immunization dose and protocol, a high level of immunogenicity with the Vip3Aa1 protein in mice was demonstrated. This relatively high level of immunogenicity was also demonstrated in rabbits (1:30,000, three doses) by Hernández *et al*.[16] and indirectly by Ben *et al*.[28]

As a result, mouse 1 was chosen for mouse hybridoma generation (1:128,000 titer) following the method described by Köhler and Milstein[18] with some modifications. As shown in **Table 2**, the total number of wells with

TABLE 1. Titration of BALB/c mouse sera immunized against Vip3Aa1 protein.

Mice	Titers vs Vip3Aa1 (before immunization)	Titers vs Vip3Aa1 (10 days after immunization)	Titers vs Vip3Aa1 (10 days after third booster)
Mouse 1	< detection limit	1:4096	1:128,000
Mouse 2	< detection limit	1:2048	1:64,000
Mouse 3	< detection limit	1:4096	1:128,000
Mouse 4	< detection limit	1:4096	1:64,000
Mouse 5	< detection limit	1:2048	1:64,000

TABLE 2. Results of the fusion and hybridoma cloning stage.

Wells Selected for Cloning	Absorbance at 492 nm	Cloning 1		Cloning 2		Cloning 3		mAb Name
		Cloning Efficiency (%)	Positive Wells (%)	Cloning Efficiency (%)	Positive Wells (%)	Cloning Efficiency (%)	Positive Wells (%)	
5	1.429	46.3	3.8	—	—	—	—	—
11	1.265	35.9	3.6	—	—	—	—	—
149	1.310	40.1	8.8	—	—	—	—	—
165	1.581	61.4	2.3	35.4	100	33.0	100	mAbcigbVip3Aa1-1
350	1.534	40.6	14.2	—	—	—	—	—
564	1.208	44.7	39.2	38.1	100	31.3	100	mAbcigbVip3Aa1-2
496	1.089	39.5	4.1	22.4	44.4	15.6	100	mAbcigbVip3Aa1-3
802	1.382	54.1	1.6	—	—	—	—	—
834	1.404	41.6	8.8	—	—	—	—	—
883	1.138	31.2	3.3	—	—	—	—	—

Total Number of Wells	Wells With Hybridomas	Fusion Efficiency (%)	Wells Positive for Vip3Aa1	Positives (%)
1056	1024	96.9	57.3	5.6

different viable cells was 1056, and 1024 of them showed viable hybridoma cells (96.9% of fusion efficiency). This high cell fusion efficiency may be explained: (1) first by the state of the myeloma cells (confluence [95%] and high viability [99%]) at the moment of the fusion; (2) the gentle procedure employed to separate cells from the spleen of the mouse; and (3) primarily by the lymphocyte:myeloma ratio (10:1). It cannot be attributed to the myeloma cell type (X63Ag8.653) used and chemical conditions such as culture medium, type and percentage (8%) of FBS, and concentration of 1450 MW PEG solution 50% (v/v) because differing results on hybridoma cloning factors have been obtained in experiments where the same chemical conditions have been used (data not shown).

Furthermore, when clones occupied 25–50% of the plate wells, supernatants were harvested and analyzed by ELISA for the measure of antibodies directed against Vip3Aa1. As a result, the total number of wells with hybridomas able to recognize the Vip3Aa1 was 57.3, which represented a positive clone efficiency of 5.6%. The three hybridomas were obtained after three consecutive cloning steps. In the case of hybridoma 165/65/7/51 (mAbcigbVip3Aa1-1), the ability or capacity to recognize the Vip3Aa-1 changed from 2.3% to 100%; whereas this parameter changed from 4.1% to 100% for hybridoma 564/24/60/8 (mAbcigbVip3Aa1-2); and from 39.2% to 100% for hybridoma 496/46/5/2 (mAbcigbVip3Aa1-3), respectively (**Table 2**). In all cases, the cloning efficiency was ~60%, which assured that ~40% of the wells were empty of cells. Thus, the cell population was statistically closed or homogeneous. These results generally coincide with those reported by other authors who have also demonstrated the high immunogenic capacity of proteins isolated from bacteria and the efficiency of cloning in just a few steps.[29]

Subsequently, the three hybridomas, mAbcigbVip3Aa1-1, mAbcigbVip3Aa1-2, and mAbcigbVip3Aa1-3, were purified from the ascites fluid of BALB/c mice. The respective ascites were harvested from mouse intraperitoneal cavities, filtered under sterile conditions, and applied in individual chromatographic columns packed with Protein A Sepharose Fast Flow, as described earlier. It is well known that protein A is a cell wall component of the *Staphylococcus aureus* bacterium routinely used for the purification of a great variety of antibodies. The binding between these two proteins is established between the Fc region of the antibody domain and the C-terminal region of staphylococcal protein A.[30] From these three hybridomas, antibodies were eluted from the columns with 100 mM citric acid, pH 6.0. The elution of antibodies at lower pH values was not detected in any case, and one of the respective affinity chromatograms is illustrated in **Figure 1**. On the other hand, the results of antibody purification by affinity chromatography (the antibody capture step), SEC, concentration, and filtration under sterile conditions

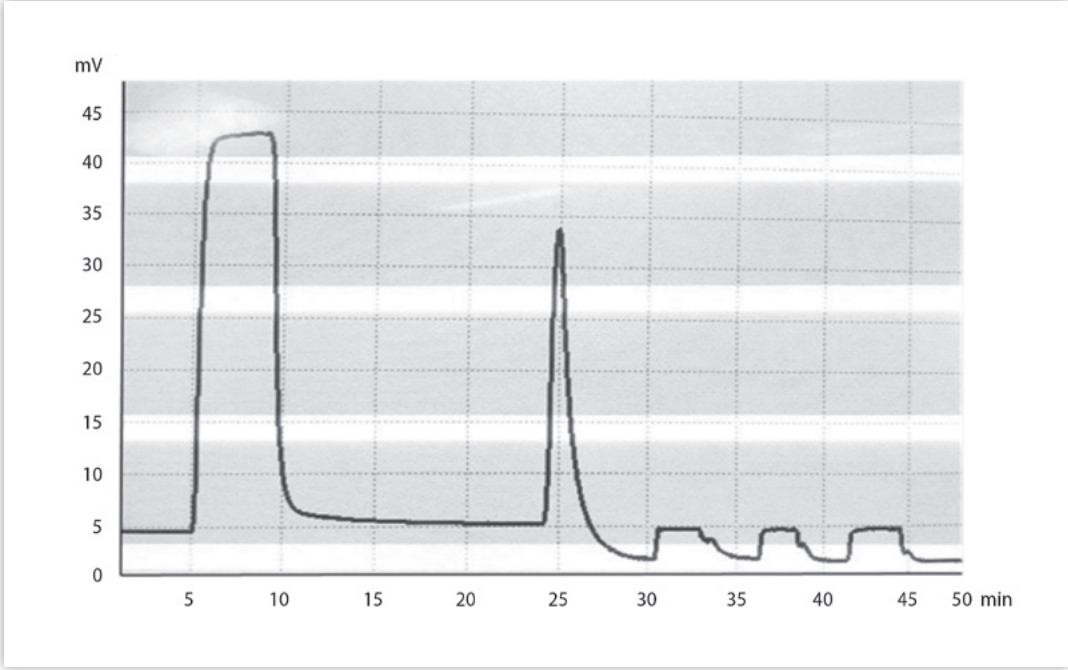


FIGURE 1. Purification by Protein A Sepharose affinity chromatography of mAbcigbVip3Aa1-1, mAbcigbVip3Aa1-2, and mAbcigbVip3Aa1-3. In all cases, the first fraction corresponded with the non-bound fraction, and the rest of the fractions corresponded with elution fractions in 100 mM citric acid at pH values of 6.0, 5.0, 4.0, and 3.0, respectively. One representative chromatogram has been shown.

are summarized in **Table 3**. The yield, expressed as the amount of antibodies per volume of ascites, ranged from 2.4–4.9 mg/mL with the maximum value reached with mAbcigbVip3Aa1-3. The recovery with affinity chromatography was ~70% for all cases, which coincides with recovery values reported by other authors for the purification of low protein A binding mouse IgG1.[30] In terms of recovery, the behavior of the SEC step performed to exchange the affinity chromatography elution buffer to 20 mM Tris/150 mM sodium chloride, pH 7.6, was also similar (95.0%/105.3%/93.0%) to those reported by the equipment manufacturer (~95%). However, an underestimation of the mAb yield was detected for the mAbcigbVip3Aa1-1 in the affinity chromatography and SEC steps because the amount of antibody increased after the concentration step. The concentration step was the least effective, showing recoveries ranging from 68.1–135.2%. There was no explanation for this relatively low recovery, although interactions of the antibodies with the 10 kDa ultrafiltration membrane must have taken place because no antibody was quantified in the permeated solution of the ultrafiltration process. To summarize the purification processes, recoveries ranged from 48.5–88.6%.

The purities of mAbcigbVip3Aa1-1, mAbcigbVip3Aa1-2, and mAbcigbVip3Aa1-3 were measured by the combination of an SDS-PAGE and a densitometry analysis. In the SDS-PAGE performed under reducing conditions, two bands were detected in all cases, 50 and 25 kDa. Both bands corresponded with the heavy and light chains of the IgGs. On the contrary, only a majority band around 150 kDa was detected in the non-reducing conditions by SDS-PAGE, which also corresponded with the SDS-PAGE pattern of the non-reduced IgGs where several isoforms were observed around this molecular weight (**Figure 2A,** following page). The reducing condition purity results shown by SDS-PAGE were 97.3% for mAbcigbVip3Aa1-1, 96.2% for mAbcigbVip3Aa1-2, and 96.5% for mAbcigbVip3Aa1-3 (**Table 3**). Similar results were estimated in the non-reducing conditions by SDS-PAGE. The HPLC-GF analysis also demonstrated the high purity of the respective mAb molecules, showing a chromatographic profile characterized by only one peak or fraction at the same residence time (high molecular homogeneity of monomers+dimers) (**Figure 2 B–D,** following page). The average HPLC-GF chromatography purity was estimated to be 99.2 ± 2%, which was a consequence of the high selectivity of the Protein A Sepharose affinity chromatography, the extensive washing of the column, the elimination of non-immunoglobulin contaminants, and the elution conditions

TABLE 3. Summary of mAb purification of Vip3Aa1 protein.

mAb Name	Steps	Volume (mL)	mAb Yield (mg/mL)	mAb Amount (mg)	mAb Recovery (%)	SDS-PAGE Purity (%)	HPLC-GF Purity (%)
mAbcigbVip3Aa1-1	Ascites	19	2.4	46.1	—	—	—
	Affinity Chromatography	34	—	32.3	70.1	—	—
	Size-Exclusion Chromatography	68	—	30.6	95.0	—	—
	Concentration and Filtration	9	—	41.4	135.2	97.3	99.2
	Total mAb Recovery: 88.6%						
mAbcigbVip3Aa1-2	Ascites	27	4.3	116.7	—	—	—
	Affinity Chromatography	40	—	82.8	71.0	—	—
	Size-Exclusion Chromatography	80	—	87.2	105.3	—	—
	Concentration and Filtration	13	—	59.4	68.1	96.2	97.1
	Total mAb Recovery: 50.8%						
mAbcigbVip3Aa1-3	Ascites	21	4.9	104.3	—	—	—
	Affinity Chromatography	39	—	74.1	71.0	—	—
	Size-Exclusion Chromatography	75	—	68.1	93.0	—	—
	Concentration and Filtration	11	—	50.6	74.3	96.5	97.3
	Total mAb Recovery: 50.8%						

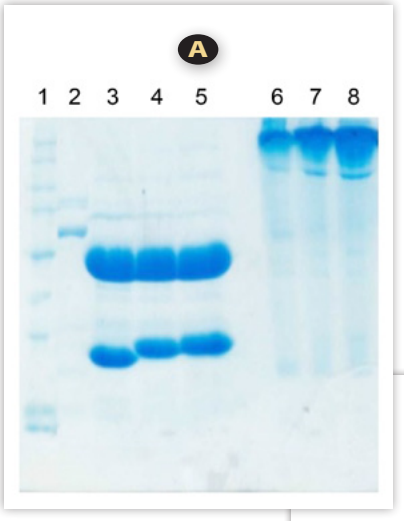
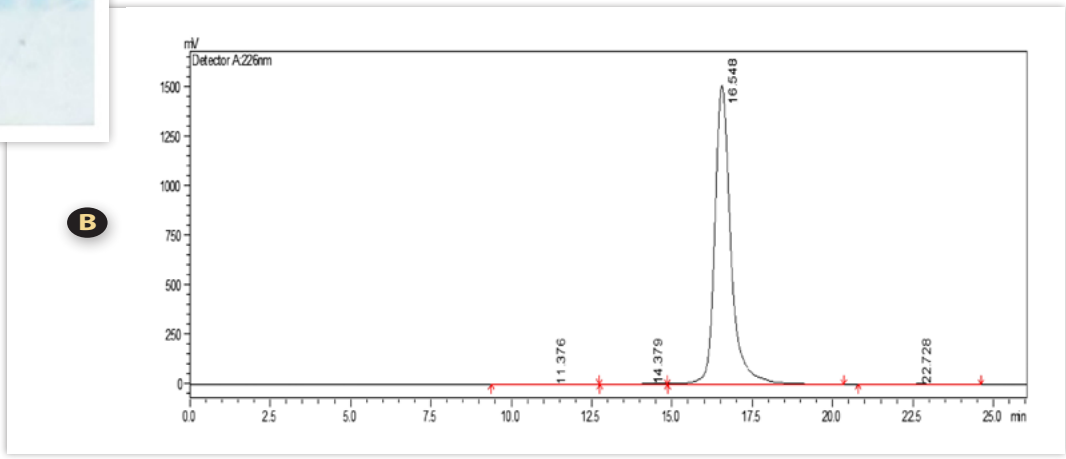
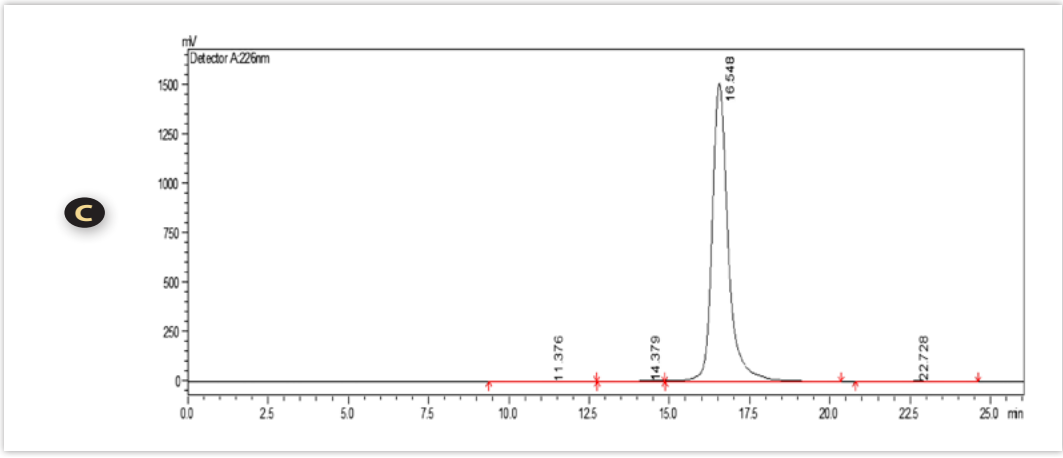


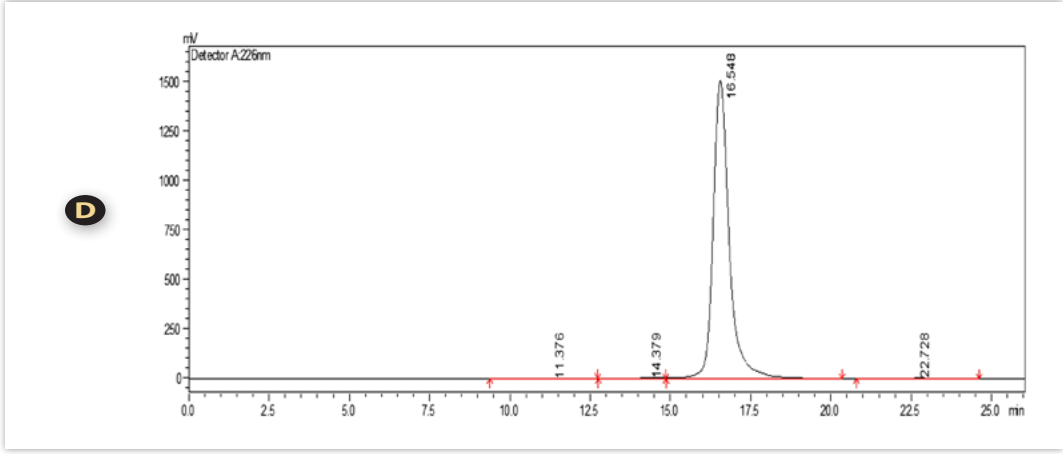
FIGURE 2.

(A) Purity of the mAbs following purification, and estimated by SDS-PAGE (12%); **Lane 1:** molecular weight markers (Broad Range Protein, Promega); **Lane 2:** Vip3Aa1; **Lane 3:** mAbcigbVip3Aa1-1 (under reducing conditions); **Lane 4:** mAbcigbVip3Aa1-2 (under reducing conditions); **Lane 5:** mAbcigbVip3Aa1-3 (under reducing conditions); **Lane 6:** mAbcigbVip3Aa1-1 (under non-reducing conditions); **Lane 7:** mAbcigbVip3Aa1-2 (under non-reducing conditions); and **Lane 8:** mAbcigbVip3Aa1-3 (under non-reducing conditions).

(B) HPLC-GF chromatogram of the mAbcigbVip3Aa1-1; **(C)** HPLC-GF chromatogram of the mAbcigbVip3Aa1-2; and **(D)** HPLC-GF chromatogram of the mAbcigbVip3Aa1-3.







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- New adjuvants for biopharmaceuticals and veterinary products
- Formulation screening and stability testing for biopharmaceuticals and veterinary products

Symposium 4: *Biocontrol Formulation*
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- Process analytical technologies (PAT)

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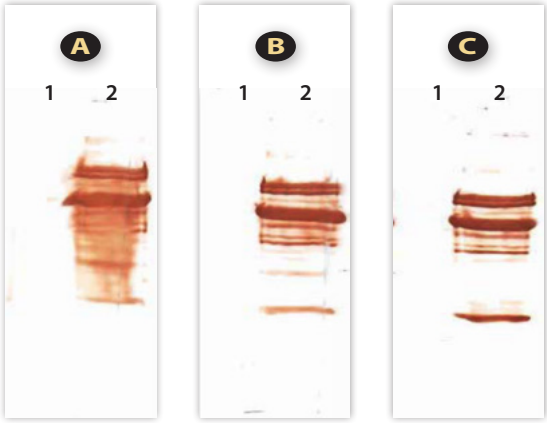

FIGURE 3. Results of the Western blot performed to demonstrate the recognition of sequential or continuous epitopes in the Vip3Aa1 with: **(A)** mAbcigbVip3Aa1-1; **(B)** mAbcigbVip3Aa1-2; and **(C)** mAbcigbVip3Aa1-3. **Lane 1:** BSA (negative control); and **Lane 2:** Vip3Aa1.

at pH 6.0. Other mouse IgGs eluted from the Protein A Sepharose affinity matrix at lower pH values.

To corroborate whether the epitope recognized by the mAb directed against Vip3Aa1 was sequential or conformational, a Western blot assay was performed. In this assay, the Vip3Aa1 protein was first applied in an SDS-PAGE and then individually faced to the mAbcigbVip3Aa1-1, mAbcigbVip3Aa1-2, and mAbcigbVip3Aa1-3. BSA was used as a negative control. Results demonstrated the specific recognition of the Vip3Aa1 protein for each mAb, which corroborated that the epitope recognized by these mAbs is sequential or continuous (**Figure 3**). The Vip3Aa1 bands identified by mAb were at 88 kDa (pro-toxin) and at 65 kDa (active toxin). Other less-represented bands were also identified, which should correspond with degradation fragments of the endotoxin. No reaction with BSA was detected in any of the mAb assays.

The IgG subclass determination analysis evidenced that these three mAbs were IgG1. This analysis was done by ELISA using labeled antibodies specific for mouse IgG1 and mouse IgG2a. Other labeled antibodies were not used because the previous results obtained in the mAb purification by Protein A Sepharose affinity chromatography suggested that these mAb molecules were mouse IgG1 (**Figure 4**). The highest percentage of immunoglobulin subclass produced in mouse is IgG1, so this would usually be expected.[31]

To complete the mAb characterization, the respective association constants by the Vip3Aa1 endotoxin were also measured. This determination was performed using the method described by Beatty *et al.*[22] and the mathematical formula $K_{aff} = n - 1/2\,(n[Ab'] - [Ab])$. The absorbance values

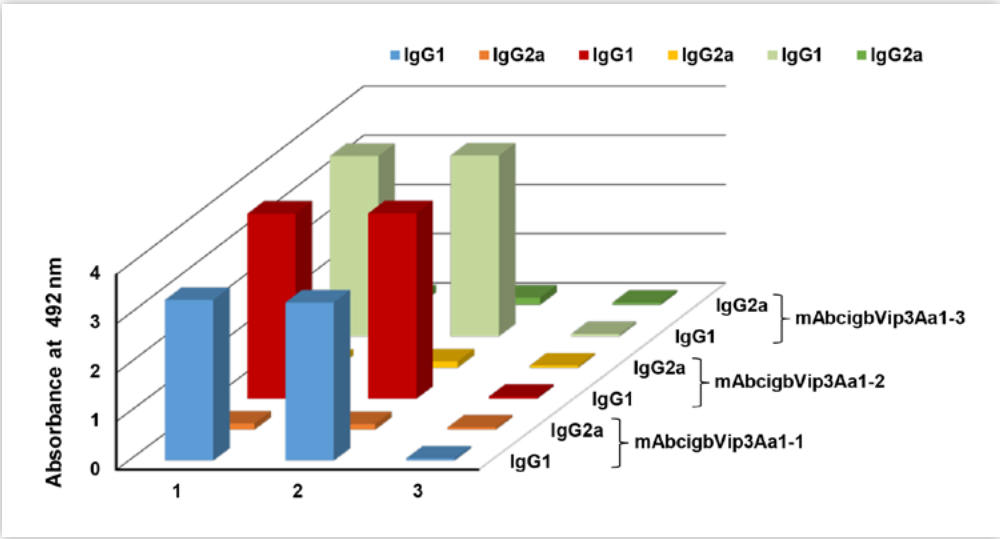

FIGURE 4. Results of mAb isotype determination: **(1)** Vip3Aa1 protein (at 10 µg/mL); **(2)** Vip3Aa1 protein (at 5 µg/mL); and **(3)** negative control (Vip3Aa1 at 10 µg/mL + respective conjugates).

identified as Ab and Ab' appear to be represented in **Figure 5**. Results revealed: K_{aff} (mAbcigbVip3Aa1-1) = 1.75×10^{-9} M; K_{aff} (mAbcigbVip3Aa1-2) = 1.26×10^{-10} M; and K_{aff} (mAbcigbVip3Aa1-3) = 1.41×10^{-10} M. It was also expected because the application of several boosters with T cell-dependent antigens such as proteins during the immunization of the animals usually leads to the maturation of the mAb association-constant genes and thus, the generation of mAbs with high or very high K_{aff}.

The immunochemical tool (sandwich ELISA) developed in this study using the technology of mAb production in the mouse allowed the detection and quantification of Vip3Aa1 purified from *E. coli*. As described in the Materials and Methods section, the mAbcigbVip3Aa1-1 was used as the Vip3Aa1 protein-capturing antibody at 10 µg/mL, whereas mAbcigbVip3Aa1-2 was employed as the labeled

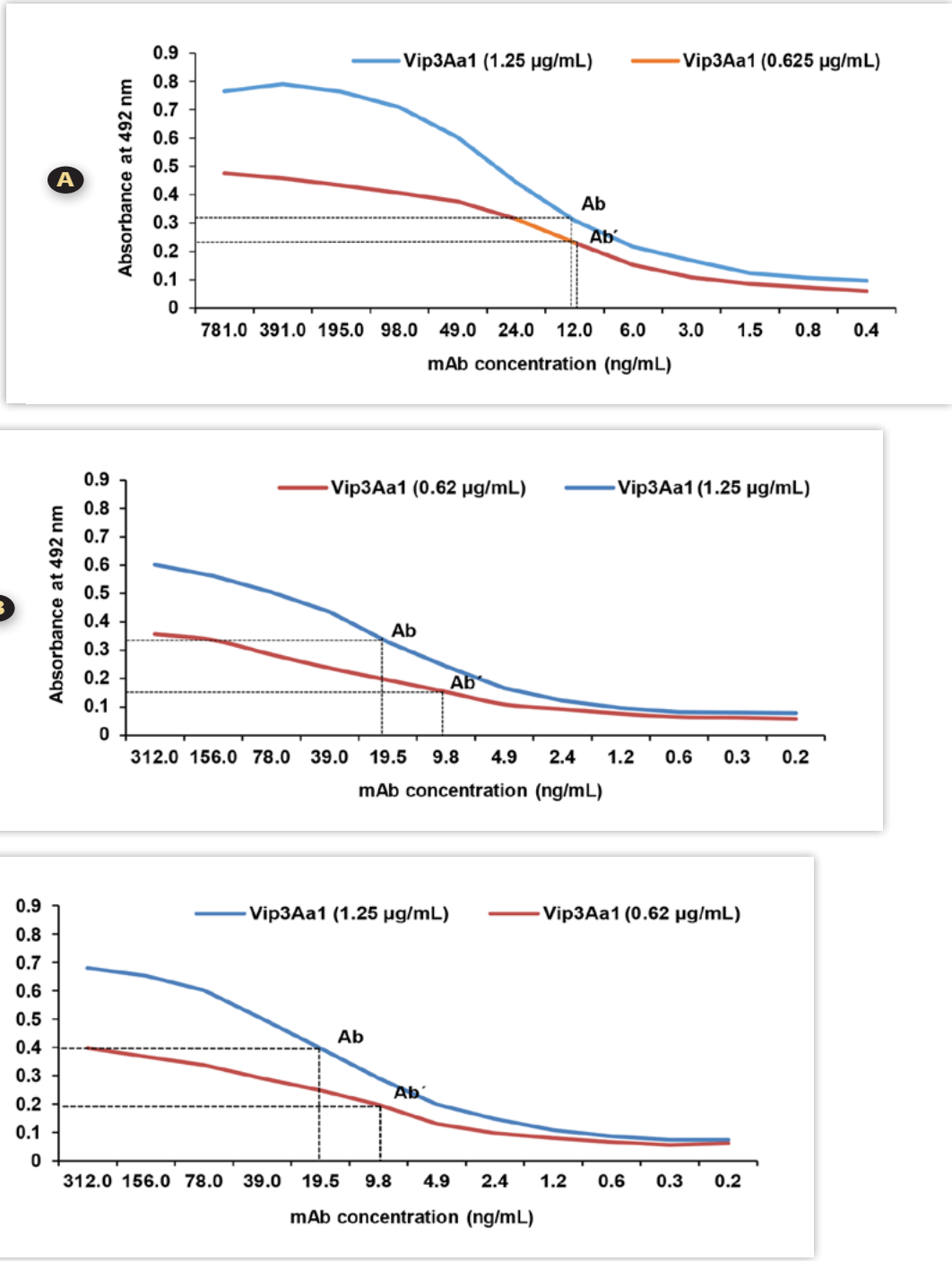

FIGURE 5. Illustration of the ELISA performed to determine the association constant of the mAb directed against Vip3Aa1: **(A)** mAbcigbVip3Aa1-1; **(2)** mAbcigbVip3Aa1-2; and **(3)** mAbcigbVip3Aa1-3.

antibody at a dilution of 1:5000. Another ELISA version using mAbcigbVip3Aa1-3 as a coating antibody was also evaluated. In this case, results were similar to those obtained with mAbcigbVip3Aa1-1. It was demonstrated that either the epitope recognized by the mAbcigbVip3Aa1-2 and mAbcigbVip3Aa1-3 is different than the epitope recognized by mAbcigbVip3Aa1-1 or that the epitope is repeated in the amino acid sequence of the Vip3Aa1 protein. In this detection and quantification system, the Vip3Aa1 recombinant protein produced in the JM109 *E. coli* strain, and used as an antigen for mAb generation, was always used as a positive control of the antibody recognition capacity and also as the standard curve of the assay. In this sense, **Figure 6** summarizes some properties of the ELISA based on mAbs. For instance: the total time of the assay was 3.5 h; the background estimated as the mean ± 2 standard deviation (SD) of the ELISA negative control was 0.050 of absorbance at 492 nm; the linear range of the standard curve ranged from 12.5–800 ng/mL; $R^2 = 0.9987$; and 12.5 ng/mL and 25.0 ng/mL were the detection and quantification limits, respectively. Therefore, the detection system was sensitive enough to detect this recombinant protein expressed in transgenic plants but less sensitive to the ELISA based on pAbs (detection limit: 1 ng/mL). However, the quantification limits were almost similar for both ELISA types (25 ng/mL for mAb-ELISA and 30 ng/mL for pAb-ELISA).[16]

It was necessary to come up with an answer to the problem found with the system based on pAbs directed against Vip3Aa1 where a non-specific reaction was detected against some components of corn plant leaves. The mAb-based sandwich ELISA assay dealt with the extracts of macerated leaves of the non-transgenic plants and event TC1507 transgenic corn plants expressing the Cry1Fa1 endotoxin. **Figure 7** shows the results of the ELISA test developed for detecting Vip3Aa20 in event MIR162 transgenic corn plants. It was evident that the system was

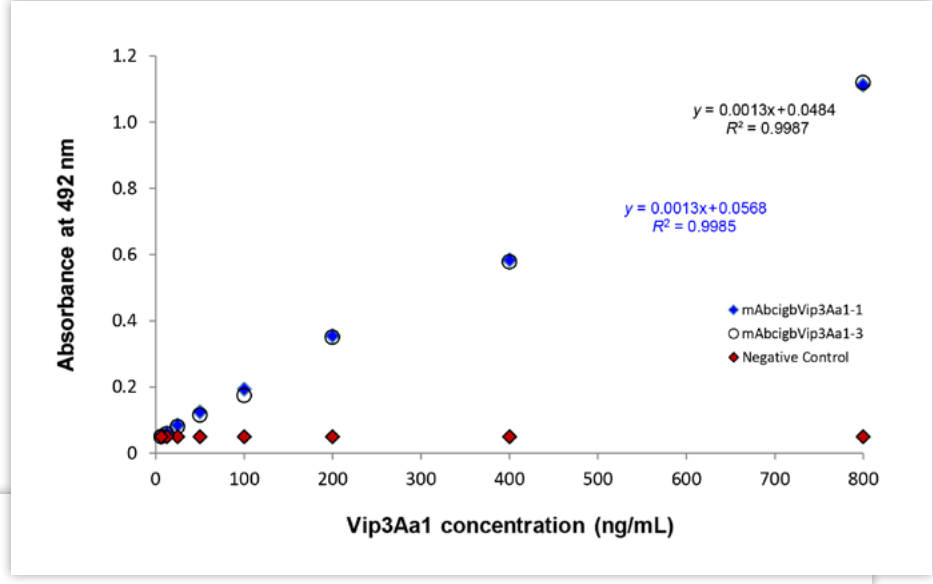

FIGURE 6. Results of the ELISA assay performed to quantify Vip3Aa1 using mAbcigbVip3Aa1-1 and mAbcigbVip3Aa1-3 as Vip3Aa1 capturing antibodies (10 µg/mL), respectively. The conjugate used was the mAbcigbVip3Aa1-2 horseradish peroxidase (1:5000). Negative control: all components of the ELISA without Vip3Aa1 (n=3).

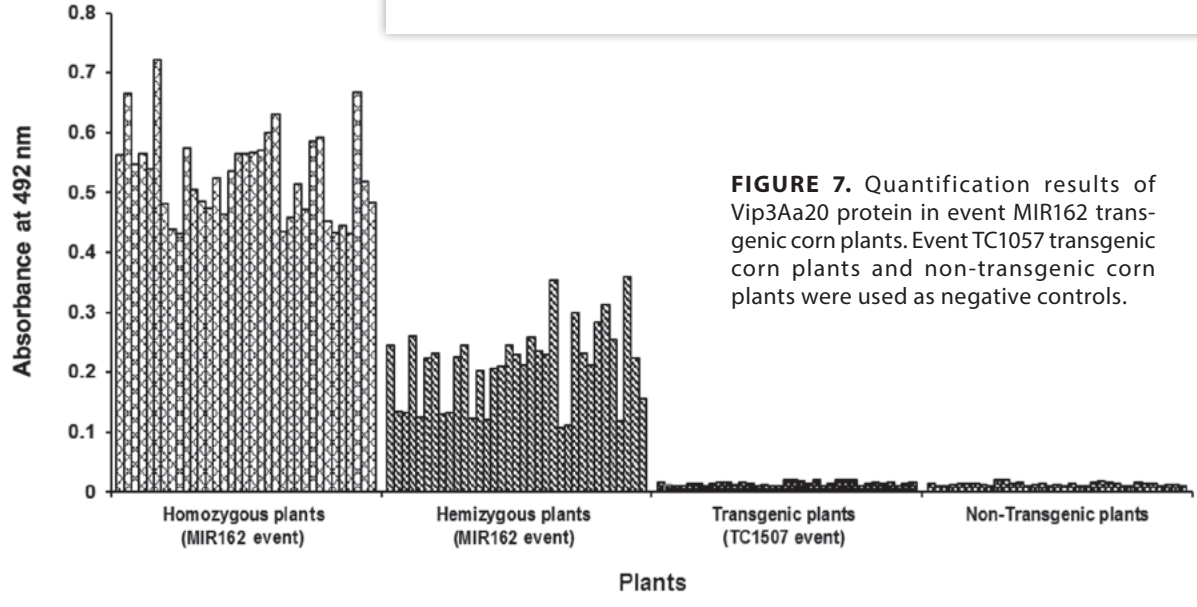

FIGURE 7. Quantification results of Vip3Aa20 protein in event MIR162 transgenic corn plants. Event TC1057 transgenic corn plants and non-transgenic corn plants were used as negative controls.

able to detect event MIR162 plants that expressed the Vip3Aa20 protein. Whereas, there was no reaction with components of non-transgenic plants and event TC1507 transgenic corn plants, at least beyond the detection limit of the ELISA. The average values of absorbance with samples corresponding to the non-transgenic plants and event TC1507 transgenic corn plants were 0.014 ± 0.02 and 0.015 ± 0.03, respectively which is far below the background of the immunoassay (0.05). This undoubtedly corroborated the specificity of the system developed on mAbs and successfully achieved the goals of this study.

Furthermore, we extended the applicability of the system to the discrimination between homozygous (double gene copy) and hemizygous (one gene copy) with event MIR162 transgenic corn plants. For this purpose, four groups of plants classified by real-time PCR were included in the study composed of: 35 event MIR162 transgenic corn plants (double gene copies), 35 event MIR162 transgenic corn plants (single gene copy), 35 event TC1507 transgenic corn plants, and 35 non-transgenic corn plants. This portion of the study was conducted because it is important to know the correlation between the number of the specific gene copies and the level of the target protein expression. This helps in choosing homozygous parental transgenic plants to then be cross-bred with corn plants adapted for growth under specific regional environmental conditions. This is of great importance because the crops of hemizygous plants can promote an insect-resistance phenomenon. If transgenic plants show a relatively low level of Vip insecticidal protein expression, the survival of insects resistant to the protein is a real possibility. As **Figure 6** illustrates, there were significant differences among the two event MIR162 transgenic corn plants (homozygous/two gene copies, and hemizygous/single gene copy), $p = 0.000$. The average value of the Vip3Aa20 measured in plants with two gene copies was 241.7 ± 43.2 ng/mL (6 µg/g fresh weight), while 51.7 ± 40.8 ng/mL (1.29 µg/g fresh weight) was estimated for transgenic corn plants with only one gene copy.

We have concluded that the development and application of the event MIR162 transgenic corn plant detection and quantification system is promissory for further development work on transgenic plant characterization. It is capable of high-capacity sample processing, has an overall simplicity with a wide distribution of knowledge using the ELISA system, combines qualitative and semi-quantitative formatting, can be used onsite, and is lower in cost compared with real-time PCR and the estimation of target protein concentration. Obviously, all of these advantages were validated from the moment in which the specific mAb was generated.

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Conflict of Interest

None of the authors have a conflict of interest with the publication of these results.

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