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Control of mosquito larvae with TMOF and 60 kDa Cry4Aa expressed in *Pichia pastoris*

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Abstract: Cry4Aa 678 amino acids fragment (60 kDa) was cloned into *Escherichia coli*. After induction with IPTG the 60 kDa Cry4Aa fragment was purified by Ni chromatography, separated by SDS PAGE and identified by mass spectrometry (MS/MS). The 60 kDa Cry4Aa fragment exhibited high toxicity towards *Ae. aegypti* larvae. The earlier results [1] show that *Pichia pastoris* yeast cells expressing *tmfA* (synthetic gene coding for the Trypsin Modulating Oostatic Factor of *Ae. aegypti*) together with *E. coli* cells expressing *Bti* toxin genes (*cry4Aa, cry11Aa, cyt1Aa* and *p20*) are synergistic. Therefore, *P. pastoris*, which synthesizes high amounts of heterologous proteins was genetically engineered to produce TMOF and Cry4Aa. Codon-optimized synthetic genes, *cry4Aa-tmfA*, *gst-cry4Aa-tmfA, tmfA* and *gfp-tmfA* that were expressed by *P. pastoris* and fed to *Ae. aegypti* larvae caused 90% mortality. GST (glutathione-S-transferase) enhanced the activity of Cry4Aa-TMOF and protected it from heat denaturation and GFP (Green Fluorescent Protein)-TMOF allowed us to follow yeast cells consumption by individual larva using fluorescent microscopy. This report shows for the first time that 60 kDa Cry4Aa and TMOF expressed together in *P. pastoris* are highly toxic to *Ae. aegypti* larvae.

Keywords: TMOF, 60 kDa Cry 4Aa, mosquito larvae*, Pichia pastoris,* genetic engineering, bio-control

INTRODUCTION

Mosquitoes transmit many important diseases such as malaria, dengue and encephalitis, that cause misery death and economic burden. Using chemical

insecticides to control mosquito populations often cause ecological problems and rapidly induce resistance in treated mosquito populations [1]. Therefore, an effective mosquito control strategy has to use biological insecticides that do not promote resistance or cause damage to the environment.

A mosquito decapeptide hormone with biological insecticide, Trypsin Modulating Oostatic Factor (TMOF) [2], that inhibits the biosynthesis of trypsin- and chymotrypsin-like enzymes in the midgut epithelial cells of female and larval mosquitoes [3, 4] has been proposed as a biological insecticide against mosquitoes [5]. In adults, TMOF causes inhibition of blood digestion that causes inhibition of egg development (sterility). In larvae TMOF causes inhibition of food digestion in the gut, anorexia, starvation and death [4, 5]. Because TMOF easily traverses the gut epithelial cells and enters the hemolymph it binds TMOF specific receptor(s) on the epithelial cells, and stops trypsin biosynthesis and egg development [4-8]. TMOF that was expressed on the coat protein of Tobacco Mosaic Virus and in yeast cells and was fed to mosquito larvae caused anorexia and death [4, 8]. Thus, orally fed TMOF can be used as an effective larvicide against many mosquito species [4, 9]. Synthetic TMOF formulated to control mosquito larvae is not practical, because the hormone is readily soluble, and mosquito larvae are filter feeders [10]. On the other hand, expressing TMOF in bacterial, algal and yeast cells that are eaten by larvae is a simple, efficient and safe way to control mosquitoes [1, 8].

Bacillus thuringiensis subsp. *israelensis*(*Bti*) Cry toxins are *per os* poisons that have been used as effective and safe biological control for over 30 years against larvae of many mosquito and black fly species [11, 12]. Although extensive use of *Bti* in the last 25 years generally did not cause resistance in field populations of mosquitoes [13, 14], resistance has been reported in field populations of *Culex pipiens* and in laboratory populations of *Cx. Quinquefasciatus* if one gene (*Cry4Aa*) was used, however, the resistance was reversed in the presence of Cyt1Aa [29, 30].

Current formulation of *Bti* does not allow it to persist in the field for long durations [12, 17], thus expressing *Bti* toxins in organisms that are readily eaten by mosquito larvae is a possible solution [15, 16]. Genetic engineering of cyanobacteria with a combination of *Bti* genes (*cry4Aa, cry11Aa* and p20) has been reported [18-20] showing that the expressed genes were highly toxic to mosquito larvae. Recently Borovsky et al. [1] reported that TMOF and the δ-endotoxin Cry11Aa of *Bti* are highly toxic and synergistically kill mosquito larvae. This study explores the possibility of expressing *tmfA* and a truncated *cry 4Aa* in *P. pastoris* cells as a future biological control agent.

MATERIALS AND METHODS

Cloning and expression in bacteria

A 2035bp DNA (Figure 1c) was amplified by PCR from a *cry4Aa* DNA (3543 bp) and cloned into pCR2.1 (Invitrogen, Carlsbad, CA). A*cry4Aa* 2035 bp amplicon was PCR amplified using forward DB 1000 (GGGGGGATCCGCCAATAGAAAATAGTCCAAAACAATTATTAC) and backward DB 1001 (GGGGCTGCAGTCATTGTTGTACTGTTTC-TAATTTTTGT) primers containing *Bam*HI and *Pst*I restriction sites, respectively. The amplicon similar in size to a 60 kDa Cry4Aa reported earlier [24] was ligated into pCR XL TOPO (Invitrogen, CA USA),amplified in *E. coli*, harvested and purified with QIAprep minispin column (Quiagen, CA USA). The purified plasmid (5 µg) was cut with *Bam*HI and *Pst*I (5 units each), DNA separated by agarose electrophoresis and a DNA band (2000 bp) was cut, purified and ligated into pETduet and pET41a(+). The later expresses GST as a fusion tag protein (Novagen, Madison, WI) (Figures 1c, d). After ligation, INVαF' cells were transfected and spread on agar plates containing carbenicillin and colonies were analyzed by PCR for *cry4Aa*. Plasmids with inserts harvested, sequenced and plasmids carrying the right insert (2035bp) were cloned and expressed in BL21 star cells in the presence of IPTG (1 mM) for 4 and 24 h (Novagen, Madison, WI).

Cloning and Expression in *P. pastoris*

A Zeocin-selectable plasmid pPICZB (Invitrogen, Carlsbad, CA) contains an alcohol oxidase 1 promoter (P_{AOX1}) fused to a multiple cloning site and *AOX1* termination sequence was used for cloning and expressing recombinant proteins in *P. pastoris*. KM71H and KM71 cells were induced in the presence of methanol (0.5%) (Invitrogen, Carlsbad, CA).

Recombinant genes *tmfA*, *gfp-tmfA, cry4Aa-tmfA* and *gst-cry4Aa-tmfA* sequences were optimized using *Pichia* codons (www.kazusa.or.jp) (Figures 1a, b). *Ae. Aegypti tmfA* was synthesized by Gemini Biotech (Gainesville, FL) and cloned directly into pPICZB, and *gfp-tmfA*, *cry4Aa-tmfA* and *gst-cry11Aa-tmfA* were synthesized by GenScript (Piscataway, NJ). The synthetic genes were cut with *Xho*I and *Xba*I, cloned into pUC57 and subsequently ligated into pPICZB *Xho*I and *Xba*I sites (Figure 1a, b). The cloned genes were sequenced (Applied Biosystems, Foster City, CA) and analyzed at the University of Florida Biotechnology Center. (http://www.biotech.ufl.edu/about.html). Plasmids carrying full length genes without mutations were used. *P. pastoris* cells KM71 and KM71H were transformed, grown and induced for 96 h in the presence of methanol and zeocin as described before [1].

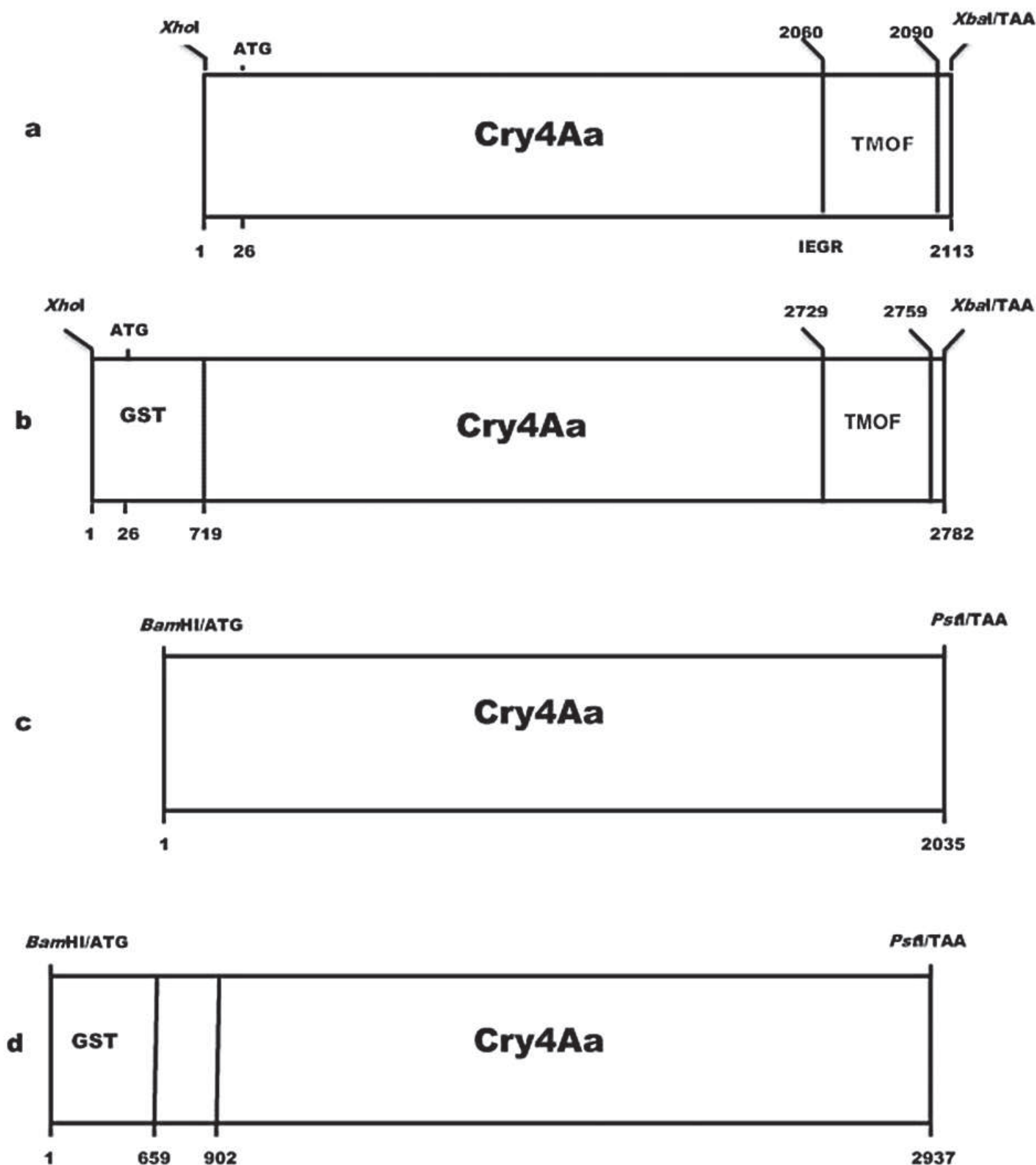

Figure 1. Genomic organization of (a) *cry4Aa-TMOF* (2113 nt), (b) *gst-cry4Aa-TMOF*(2782 nt) (c) *cry4Aa* (2035 nt)and (d) *gst-cry4Aa* (2937 nt) synthetic genes for *P. pastoris* (a and b) and *E. coli* (c and d) with restriction enzymes cleavage sites for cloning, the ATG start signal the IEGR trypsin cleavage sites at positions 2060 nt and 2729 nt for *P. pastoris* genes and the TAA stop signal.

Protein purification and mass spectrometry

Yeast or *E. coli* cells were collected by centrifugation, broken with Y-PER

and B-PER, respectively (Pierce, Rockford, IL) or with 8 M urea and glass beads in a DNA FastPrep (BIO 101, Inc., Vista, CA), centrifuged and stored frozen at -20 °C. Extracted proteins were purified and analyzed by SDS PAGE [25] and mass spectrometry as was described before [1].

Bioassays for larvicidal activity

Recombinant *E. coli* were grown at 37 °C in LB medium, induced with IPTG for 4 and 24 h [1]. Recombinant yeast cells were collected by centrifugation, washed, re-suspended in distilled water and used without or with heat inactivation at 56 °C for 3 h. Autoclaved brewer's yeast and heat inactivated Pichia cells without *tmfA* were used as controls. Washed recombinant cells were added into 160 mL sterile distilled water in glass dishes containing 20 larvae [1, 26]. Each bioassay was performed in triplicates and mortality was followed for up to12 days.

RESULTS

Larvicidal activity of 60 kDa Cry4Aa in *E. coli*

Yamagiwa et al. [24] reported that Cry4Aa 130 kDa is first truncated into a 60 kDa fragment of 695 amino acids that is further cleaved into 45 and 20 kDa fragments that associate and become toxic to mosquito larvae. These observations prompted us to clone and express a 2035 bp DNA fragment of *cry4Aa* in *E. coli* encoding 678 amino acids which is shorter than the original proposed 695 amino acids [24] to determine the minimal length of *cry4Aa* that is toxic to larvae. Two plasmids were used: pETDuet1 and pET41a(+), the latter carries a glutathione-S-transferase (*gst*) gene that was fused in frame with *cry4Aa* to enhance solubility of the truncated Cry4Aa in bacterial cells (Figure 1d). Ten-fold less bacterial cells harboring pET-41a(+) and synthesizing GST-Cry4Aa were needed to kill first instar larval *Ae. aegypti* (10^6 cells/mL compared with 10^7 cells/mL) and the larvae died much faster than those that were fed bacteria transformed with pETDuet 1 expressing Cry4Aa (Figures 2a, b, respectively). The same bacterial cells when fed together with Pichia cells (0.5 mg/mL) failed to kill the larvae (Figures 2a, b). However, if Pichia synthesizing TMOF (298 nM) cells were used all the larvae died in 9 days (Figures 2a, b). Larvae that ate GST-Cry4Aa and TMOF died faster indicating synergy between TMOF and Cry4Aa. Larvae that ate recombinant *P. pastoris* synthesizing TMOF died much slower because TMOF mode of action is different than *Bti* (Figure 2b). Controls that fed non-recombinant Pichia cells did not die (Figure 2).

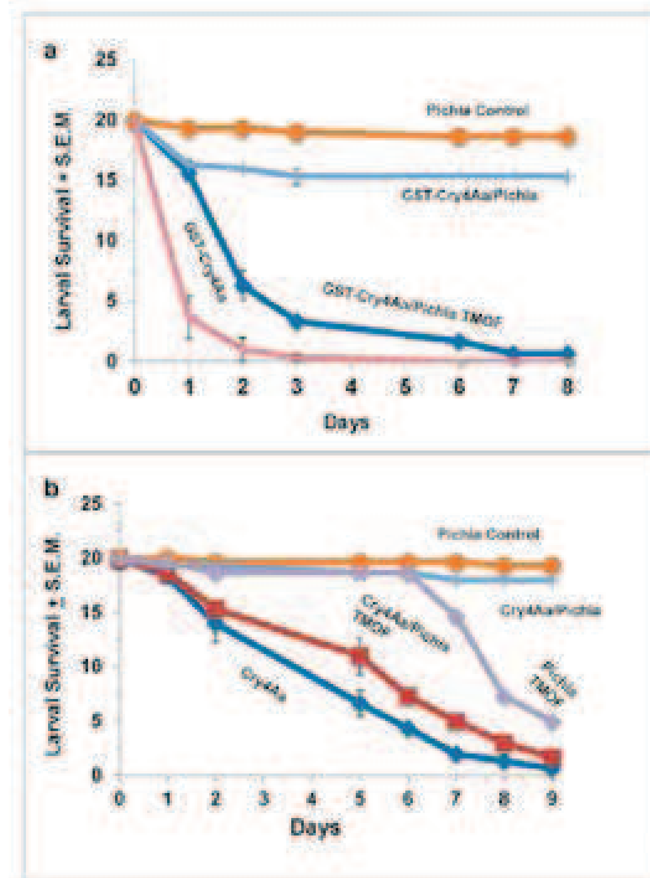

Figure 2. Feeding *Ae. aegypti*larvae mixtures of recombinant *E. coli* cells alone or with recombinant *P. pastoris* cells expressing TMOF. *E. coli* cells were transformed with: (a) pET-41a(+) expressing GST-Cry4Aa (10^6 cells/mL) and (b) pETDuet1 expressing Cry4Aa(10^7 cells/mL). Controls were fed non-recombinant *P. pastoris* cells. Each point represents the mean of 3-5 determinations ±S.E.M.

Larvicidal activity of 60 kDa Cry4Aa and TMOF in *P. pastoris*

Our earlier results prompted us to express combinations of synthetic *tmfA*, *gfp-tmfA, cry4Aa-tmfA* and *gst-cry4Aa-tmfA* in *P. pastoris* KM71 and KM71H. We hypothesized that GST synthetic gene fused in frame with 60kDa Cry4Aa-TMOF will keep the fusion protein GST-Cry4Aa-TMOF soluble and thus increase cell toxicity. After methanol stimulation (0.5% for 96 h), one fraction of the yeast cells was not treated, and another fraction was heat inactivated (56 °C for 3 h). Untreated cells and heat inactivated cells ($3x10^7$ cells/mL) were fed separately to 3-5 groups of 20 larvae in 160 mL water. Larvae that ate recombinant cells expressing *gst-cry4Aa-tmfA* and *gfp-tmfA* that were heat or non-heat treated died within 12 days (72% and 95%, respectively) (Figures 3a, b). Similar results were observed when *tmfA*was not fused to *gfp* (Figures 3a, b).

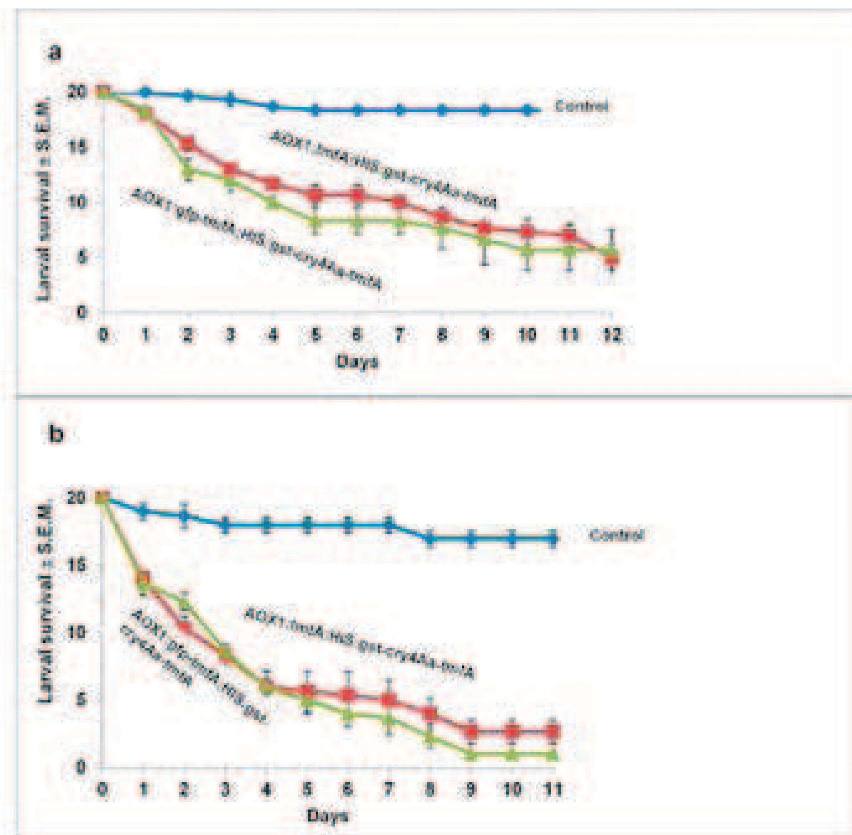

Figure 3. Feeding *Ae. aegypti* larvae recombinant *P. pastoris*($3x10^7$ cells/mL) *AOX:tmfA; His:gst-cry4Aa-tmfA* and *AOX1:gfp-tmfA;His:gst-cry4Aa-tmf*A (a) heat treated for 3h at 56 °C and (b) non-heat treated. Controls were fed non recombinant *P. pastoris* cells. Each point represents the mean of 3-5 determinations ±S.E.M.

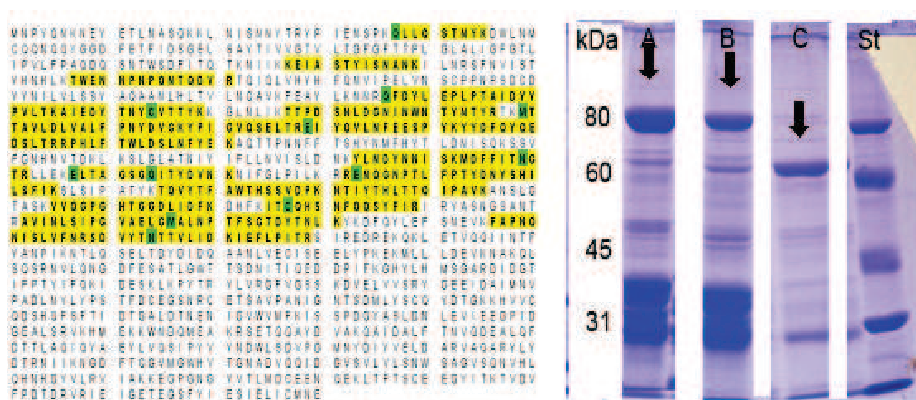

Figure 4. SDS-PAGE after Ni-NTA affinity chromatography and mass spectra identification of Cry4Aa of *E. coli* transformed with pET-41a(+) (GST-Cry4Aa) lanes A and B and pETDuet 1 (Cry4Aa) lane C. St=molecular weight marker proteins. Darker shades represent identified peptides and arrows point to protein bands that were analyzed by MS/MS.

Purification and Mass Spectrometry Analysis of Cry4Aa

To identify Cry4Aa-TMOF and GST-Cry4Aa-TMOF, *E. coli* and *P. pastoris* cells were broken, extracted with 8 M urea, and the cell extracts purified by Ni-NTA affinity chromatography. Protein (200-500 mg) eluted at 250 mM imidazole was collected and resolved by PAGE SDS [25]. After electrophoresis, gels were stained, and bands at M_r 80 kDa and 60 kDa corresponding to GST-Cry4Aa-TMOF and Cry4Aa-TMOF were cut and analyzed by mass spectrometry (Figure 4). Twenty-five unique peptides that cover 30% of the entire Cry4Aa were detected identifying Cry4Aa with 98% confidence (Figure 4). GST was also identified with 95% confidence (mass spectra not shown). Similarly, GST-Cry4Aa-TMOF band M_r 80 kDa was detected by SDS PAGE after Ni-NTA column chromatography of recombinant *P. pastoris gst-cry4Aa-tmfA* KM71H and KM71 cells. The protein band was analyzed by mass spectrometry identifying 42 unique spectra covering 65% of the entire Cry4Aa-TMOF fusion protein with 98% confidence (Figure 5). Fluorescence microscopy identified GFP-TMOF in yeast cells (results not shown) [26].

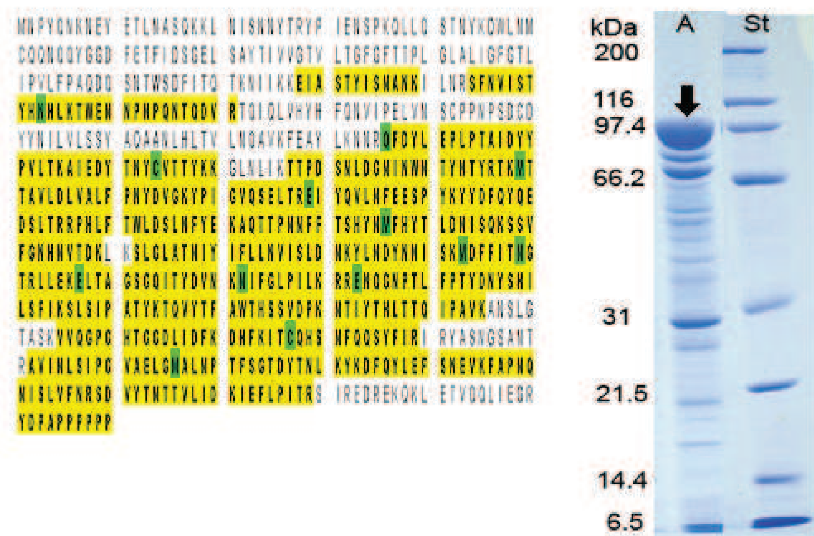

Figure 5. SDS-PAGE after Ni-NTA affinity chromatography and mass spectra identification of Cry4Aa-TMOF of *P. pastoris* transformed with *gst-cry4Aa-TMOF*. St = molecular weight marker proteins. Darker shades represent identified peptides and arrow in lane A points to the protein band that was analyzed by MS/MS.

DISCUSSION

Insect resistance to insecticides poses serious agricultural and public health problems. Thus, co-expressing several toxin genes having different modes of action might enhance toxicity and delay or prevent resistance. TMOF and δ-endotoxins kill mosquito larvae in different mechanisms, and are synergistic [1], just as δ-endotoxins from other *Bt* subsps and bacterial endochitinase against *S. littoralis* [21, 22] and *Ae. aegypti* larvae [23]. Because TMOF inhibits the biosynthesis of trypsin [2], which activates δ-endotoxins in the larval midgut, we suggest using genes that encode the active portion of these polypeptides [27] with TMOF. Borovsky et al. [1] reported that starved *Ae. aegypti* larvae are 6-35 fold more sensitive to *Bti* toxins than well-fed larvae, and starved 1st instar larvae are 10-fold more sensitive than 3rd instars. Since TMOF is more effective against 1st and 2nd instar larvae [9], the use of both toxins in early developmental stages is expected to enhance the effect of low toxin doses and reduce the amount of *Bti* required in sewage treatment ponds in which it is not effective alone.

We report here that a short chain Cry4Aa is highly toxic against mosquito larvae when fused with GST as compared with cells that expressed Cry4Aa alone (Figure 2). This toxicity was dramatically reduced in the presence of *P. pastoris* and mortality was restored only when these cells were replaced with recombinant Pichia producing TMOF. These results show that TMOF and 60 kDa Cry4Aa are also synergistic as was reported for Cry11Aa and TMOF [1]. Fusing GST and SUMO (small ubiquitin-related modifier) to proteins enhances their activity in bacteria and yeasts by increasing solubility in the cytoplasm [28]. Indeed, GST enhanced the heat stability of Cry4Aa-TMOF even at higher temperature that is 6 °C higher that was reported before [1] allowing heat inactivation of the yeast cells in future field applications. Mass spectrometry analysis of Cry4Aa-TMOF that was expressed by *P. pastoris* identified both Cry4Aa and TMOF (Figure 5) indicating that the fusion protein is not cleaved by yeast proteases and thus the fusion protein is cleaved only inside the mosquito larval gut.

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