

# Synergy Between *Aedes aegypti* Trypsin Modulating Oostatic Factor and $\delta$ -Endotoxins

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**Abstract:** Starved first instar *Aedes aegypti* larvae were 35-fold more sensitive to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) toxins than fed larvae. Feeding larvae *Pichia pastoris* yeast cells expressing *tmfA* (synthetic gene coding for the Trypsin Modulating Oostatic Factor of *Ae. aegypti*) together with *Escherichia coli* cells expressing *Bti* toxin genes (*cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20*) indicate that TMOF and Cry toxins are synergistic. *tmfA* was cloned and expressed in the cyanobacterium *Anabaena* PCC 7120 and the hormone was purified by HPLC and identified by ELISA. The amount of TMOF synthesized by *Anabaena* was low (0.5 - 1  $\mu\text{g}$  in  $10^8$  cells). *P. pastoris*, which synthesizes high amounts of heterologous proteins in the presence of methanol and is readily consumed by mosquito larvae, was genetically engineered to produce more TMOF. Codon-optimized synthetic genes, *cry11Aa-tmfA* and *gst-cry11Aa-tmfA*, that were cloned into *P. pastoris* and fed to *Ae. aegypti* larvae caused 87.5% mortality in 5 days. GST (glutathione-S-transferase) enhanced the activity of Cry11A-TMOF and protected it from heat denaturation. Cell free extracts of recombinant *P. pastoris* cells killed 40% of tested 4<sup>th</sup> instar larvae within 24 h, and mass spectra analysis confirmed that the recombinants synthesize Cry11Aa. This report shows for the first time that Cry toxins and TMOF are synergists to *Ae. aegypti* larvae when jointly fed or expressed in recombinant *P. pastoris*.

**Keywords:** TMOF, Cry toxins, mosquito larvae, *Anabaena*, *Pichia pastoris*, genetic engineering, bio-control.

## INTRODUCTION

Mosquitoes are vectors of major diseases such as malaria, dengue and encephalitis, and are nuisance in temperate zones. Mosquito control programs that solely rely on chemical insecticides often cause ecological problems and select for resistance due to frequent applications [1]. Thus, mosquito control relying on environmentally friendly insecticides is desired.

Trypsin Modulating Oostatic Factor (TMOF), a decapeptide hormone originally purified from the ovaries of female *Aedes aegypti* [2], inhibits the biosynthesis of trypsin- and chymotrypsin-like enzymes in the midgut epithelial cells of female and larval mosquitoes by a translational control mechanism [3, 4] stopping food digestion. The lack of free amino acids liberated from the blood meal in adult females or from digested proteins liberated in the larval gut causes inhibition of egg development (sterility) to adults and anorexia and death to larval mosquitoes, respectively [4, 5]. TMOF traverses the gut, enters the hemolymph, binds a TMOF specific

receptor(s) on the epithelial cells, and stops trypsin biosynthesis and egg development [4-8].

Mosquito larvae synthesize trypsin-like enzyme(s) in their gut as major digestive protease(s). TMOF expressed on the coat protein of Tobacco Mosaic Virus that was fed to mosquito larvae caused anorexia and death at a low dose (1 ng per larva) [4]. Thus, orally fed TMOF can be used as an effective larvicide against many mosquito species [4, 9]. Using a synthetic TMOF to control mosquito larvae is, however, expensive and inefficient because it is readily soluble in water, whereas mosquito larvae are filter feeders [10]. On the other hand, expressing TMOF in bacterial, algal and yeast cells that are readily eaten by larvae is a simple, efficient and safe way to control mosquitoes.

*Bacillus thuringiensis* subsp. *israelensis* (*Bti*) is the first subspecies of *B. thuringiensis* (*Bt*) that was found and used as an effective biological control agent against larvae of many mosquito and black fly species [11]. Its Cry toxins are *per os* poisons; they bind to larval midgut causing rapid death, and hence are considered safe larvicides that do not damage the environment [12]. Low probability of developing resistance has been observed in field populations of mosquitoes despite 25 years of extensive field usage [13, 14].

Field persistence of current preparations of *Bti* is unfortunately low [12], but could be improved by expressing

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phate buffer pH 8.0, and eluted with 20 mM and 250 mM imidazole. Samples from the eluted peaks were removed, dried by Speed Vac, rehydrated in 3% SDS and 5%  $\beta$ -mercaptoethanol, 50 mM Tris pH 8.8, heated for 5 min at 95°C and analyzed by 10% SDS PAGE [25]. The gels were fixed and stained with coomassie brilliant blue and bands that migrated at the same molecular weight of the recombinant proteins were cut, digested with trypsin and analyzed by mass spectrometry (<http://www.biotech.ufl.edu/about.html>).

### Bioassays for Larvicidal Activity

Recombinant *E. coli* were grown at 37°C in LB medium, induced with IPTG and harvested after 4 h [26]. Recombinant *Anabaena* were grown at 30°C in BG11 medium containing 25  $\mu\text{g ml}^{-1}$  neomycin in 250 ml flasks for 12 days, as previously described [19]. *Bti* and *P. pastoris* cells were grown in the appropriate liquid media and the latter induced with methanol (0.5%). Yeast recombinant cells were collected by centrifugation, washed and resuspended in distilled water, and used without or with heat inactivation at 50°C for 3 h.

Autoclaved brewer's yeast and heat inactivated (75°C or 50°C for 3 h) *Pichia* cells without *tmfA* were used as controls. Recombinant cells producing TMOF, GST-Cry11Aa-TMOF or Cry11Aa-TMOF were washed 3-times in sterile distilled water and added to 48 well plates, each containing a single 1<sup>st</sup>-instar *Ae. aegypti* larva in 1 ml sterile water. For large scale feedings, 20 such larvae were added into glass dishes containing 160 ml sterile water and different concentrations of toxins (Cry4Aa, Cry11Aa, Cyt1Aa and P20) produced in *E. coli* [27] or TMOF produced in *P. pastoris* [28]. Each bioassay was independently performed in triplicates. Mortality was followed for 12 days at 28°C. Values of LC<sub>50</sub> and LC<sub>90</sub> (concentration of cells that kill 50% and 90% of the exposed populations, respectively) were determined using probit analysis (EPA Probit analysis program) for 6-12 doses.

### ELISA (Enzyme Linked Immunoabsorbant Assay)

Recombinant bacteria were dried in a Speed Vac or lyophilized. TMOF-producing *P. pastoris* KM71H cells were grown in 4 L flasks and stimulated for 120 h with 0.5% methanol. Cells were washed with sterile water to remove medium and external proteins, and collected by centrifugation. To the washed cells 400  $\mu\text{l}$  of Y-PER (Pierce, Rockford, IL) was added and the cultures were gently shaken for 20 min at room temperature. The cells were disrupted by ultrasonic disintegration or with glass beads by shaking for 40 sec at speed 6 with Lysing Matrix C in a DNA Fast Prep Instrument (BIO 101, Vista, CA).

The amounts of TMOF in samples were determined by ELISA [29]. Briefly, to each well of reacti-bind maleic anhydride activated polystyrene plates (Pierce, Rockford, IL) 100  $\mu\text{l}$  of PBS buffer (0.1 M Phosphate, 0.15 M NaCl, pH 7.2) and TMOF of known concentrations (10 ng to 1.2 mg in 10  $\mu\text{l}$  PBS), pH 7.2 were added to construct a calibration curve. Similarly, proteins that were extracted from recombinant

cells were also tested. The maleic anhydride well plates were incubated by gentle shaking at room temperature for 1 h and at 4°C overnight without shaking. The solutions were then decanted from the wells, and to each well 300  $\mu\text{l}$  of a blocking solution TTBS (50 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, 3% BSA, pH 7.2) was added and incubated for 1 h. The solutions were then decanted from the wells, and to each well 100  $\mu\text{l}$  of TMOF antiserum that was diluted 1:100 in 0.05% Tween-20, PBS, 0.1% BSA (dilution buffer) was added and incubated for 1 h. The wells were washed 3-times with TTBS by gentle shaking for 5 minutes each. To each well 100  $\mu\text{l}$  of Goat anti-rabbit antibody linked to alkaline phosphatase diluted in a dilution buffer (1:15,000) was added, and incubated for 1 h. The wells were washed 3-times with TTBS and to each well 200  $\mu\text{l}$  of alkaline phosphatase liquid substrate (Sigma, St. Louis, MS) was added and incubated for 30-60 min until a yellow color developed and the absorbance was read at 405 nm in an ELISA plate reader Model EL 308 (Bio-Tek Instruments, Burlington, Vermont).

The absorbency at 405 nm was plotted against the log concentration (ng) of the TMOF standard to construct a calibration curve. The amount of TMOF in the tested recombinant cells was read directly from the linear part of the calibration curve.

### HPLC (High Pressure Liquid Chromatography)

Recombinant *Anabaena* cells were grown, harvested, lyophilized, washed and sonicated. The broken cells were centrifuged and the supernatants collected and acidified with 50  $\mu\text{l}$  TFA (trifluoroacetic acid). The acidified solution was vortexed and centrifuged at 14,000 rpm for 5 min. Supernatants were adsorbed onto Sep-Pak C<sub>18</sub> reversed-phase cartridges (Millipore, Milford, Mass), the column washed with 4 ml water, 0.1% TFA and TMOF was eluted with 100% acetonitrile, 0.1% TFA (4 ml), dried by Speed Vac at 40°C, and taken up in 100  $\mu\text{l}$  water. To the rehydrated samples (90  $\mu\text{l}$ ), TFA (10  $\mu\text{l}$ ) was added and the samples chromatographed by a high-performance liquid chromatography (HPLC) system with a Microsorb 5  $\mu\text{m}$  C<sub>18</sub> reversed-phase column (4.6 mm ID by 25 cm), a 5- $\mu\text{m}$  guard cartridge, and a gradient of acetonitrile (0-100%) in 0.1% TFA. Absorbance was followed at 220 nm at 1 absorbance unit at full scale (AUFS). Fractions (1 ml) were collected, dried by Speed Vac and rehydrated in 100  $\mu\text{l}$  PBS. Samples were analyzed by ELISA for the presence of TMOF using reacti-bind maleic anhydride activated polystyrene plates (Pierce, Rockford, IL).

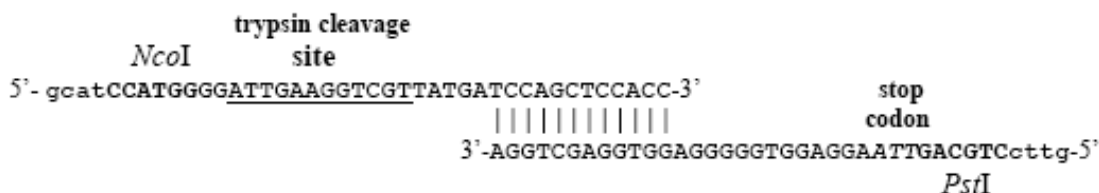
## RESULTS

### Cloning and Expression of *tmfA* in *Anabaena*

TMOF-coding region (bases 25-54, underlined; *tmfA*) was optimized using *Anabaena* PCC 7120 codons. The sequence of 12 bases coding for a trypsin cleavage site (IEGR) was introduced upstream (bases 13-24), to release in the larval midgut free TMOF that inhibits trypsin biosynthesis:

1            10            20            30            40            50            60            65  
GCATCCATGCGGATTGAAGGTCGTTATGATCCAGCTCCACCTCCCCACCTCCTTAACGCAGGAAC

To construct pBVE4-TE (Fig. 1), two overlapping oligonucleotides of 41 (*NcoI*) and 38 (*PstI*) bases with overlap of 12 nucleotides were synthesized, annealed and amplified by PCR:



The 67 bp *NcoI-PstI* amplicon was purified by PAGE, cut and ligated into pBS-SK/N at the *NcoI-PstI* site upstream *P<sub>AI</sub>* [18], and the resultant pBVE4-TE was transformed into *E. coli* XL1-Blue MRF'. pBVE4-TE was cut with *KpnI/SacI* and the *P<sub>AI</sub>-tmfA* fragment ligated into pRL488p, containing *P<sub>psbA</sub>* downstream of the *KpnI-SacI* site, yielding pRVE4-TE (Fig. 1). The recombinant plasmid was transformed into and amplified in *E. coli* XL1-Blue MRF'. The plasmid with the dual promoters *P<sub>psbA</sub>* and *P<sub>AI</sub>* was introduced into *Anabaena* PCC 7120 by biparental conjugation [24].

Another sequence encoding TMOF without IEGR was also designed for expression in *E. coli* and in *Anabaena* PCC 7120:



Two oligonucleotides, 43 bases each, were synthesized and annealed and the gene was ligated into the *PstI/NcoI* cloning site of pBS-SK/N (*NcoI* site of the vector was filled-in by Klenow fragment), yielding pBVE4-T that was transformed into *E. coli* XL1-Blue MRF'. pBVE4-TE and pBVE4-T were digested with *KpnI/SacI* and the *P<sub>AI</sub>-tmfA* fragments ligated into pRL488p, containing *P<sub>psbA</sub>* downstream the *KpnI-SacI* site, yielding pRVE4-TE and pRVE4-T, respectively (Fig. 1). The recombinant plasmids were transformed and amplified in *E. coli* XL1-Blue MRF'. The plasmids with the dual promoters *P<sub>psbA</sub>* and *P<sub>AI</sub>* were introduced into *Anabaena* PCC 7120 through DH10B (carrying conjugal and helper plasmids) by biparental conjugation [24].

To find out the expression level of *tmfA* alone (without *Bti* toxin genes) in *Anabaena*, it was fused in frame to the C-terminal segment of *p20* (encoding an accessory, non toxic *Bti* protein) and the gene was expressed with and without a protease cleavage site (IEGR). Recombinant cells were grown, and protein extracted and analyzed by HPLC and ELISA for TMOF. Transgenic clones of *Anabaena* PCC 7120 synthesized 0.5-1  $\mu$ g of TMOF in  $10^8$  cells, higher than in *E. coli* (35-650 ng in  $10^8$ - $10^9$  cells). However, when *tmfA* was expressed in *Bti* the synthesis of the hormone was even lower (67-267 ng in  $10^8$ - $10^9$  cells). These results indicate that the expression level of *tmfA* in *Anabaena*, *E. coli* and *Bti* is low. Perhaps the decapeptide precipitates in inclusion bodies or it needs to be fused to a larger protein in order to efficiently be synthesized in bacteria.

## *Bti* $\delta$ -Endotoxins and TMOF

It is anticipated that starved mosquito larvae are more sensitive to  $\delta$ -endotoxins than well nourished larvae because they are weaker, swim slower in water and may consume

more toxin than non starved larvae (Borovsky, unpublished observations). Indeed, 1<sup>st</sup> instar larvae starved from emergence were 35-fold more sensitive than well nourished larvae fed with Brewer's yeast (80 mg ml<sup>-1</sup>), whereas 3<sup>rd</sup> instar larvae that were starved of Brewer's yeast for a day were only 6-fold more sensitive to  $\delta$ -endotoxins than well nourished larvae (Table 1).

**Table 1. Toxicity of *Bti* Against *Ae.aegypti* Larvae**

Larval Instar	LC <sub>50</sub> (ng ml <sup>-1</sup> )	
	Starved	Fed
First	1.6	55.6
Third	17.7	105.5

*Aedes aegypti* larvae (2 groups of 20 larvae per group) were fed a dry preparation of *B. thurengiensis* subsp. *israelensis*  $\delta$ -endotoxins and mortality was determined after 24 h. Results are average of 2 determinations with no more than 5-10% differences between groups.

These results encouraged us to express a synthetic *tmfA* in *P. pastoris* KM71H. The yeast cells were heat inactivated (50°C for 3 h) and 3 different concentrations of the hormone were fed together with  $10^5$  *E. coli* cells expressing *cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20* [27], to groups of twenty 1<sup>st</sup> instar *Ae. aegypti* larvae, and larval survival was monitored daily for 6 days (Table 2). All the larvae that were fed by the transgenic *E. coli* alone died, but none died upon addition of *P. pastoris* cells transformed with empty pPICZB, as in the control. Thus, *P. pastoris* not only serves as a good food source for the larvae—it protects them from the *Bti* toxins as well. Feeding recombinant *P. pastoris* cells with high TMOF concentration alone (298 nM) did not cause appreciable larval mortality in 6 days. After 12 days of feeding, however, 90% of the larvae died from starvation and lack of trypsin biosynthesis (data not shown). Lower TMOF concentrations (75 nM) in the transgenic *P. pastoris* cells restored the toxicity of the  $\delta$ -endotoxins produced by *E. coli* ( $10^5$  cells); mortality reached 95% after 6 days of feeding (Table 2). Feeding *E. coli* expressing *Bti* toxin genes in the presence of *P. pastoris* cells or *P. pastoris* expressing *tmfA* alone did not affect mosquito larvae (Table 2). These results indicate that TMOF enhances the effect of *Bti* toxins in organic rich environments that are abundant in nature.





**Table 3. Feeding Larvae Cell Free Extract of *P. pastoris* Cells Expressing *gst-cry11Aa-tmfa***

Cells Extract	N	Dead larvae $\pm$ S.E.M.	Mortality (%)
a. <i>Pichia gst-cry11Aa-tmfa</i> 96 h fermentation	8	6.38 $\pm$ 0.86	40
b. <i>Pichia</i> control	4	1.0 $\pm$ 0	6

*P.pastoris* cells were fermented for 96 h and the cells were broken with glass beads and Y-PER, the extract was centrifuged, the supernatant collected and aliquots of (5.69  $\mu$ g) fed to individual early 4<sup>th</sup> instar *Ae. aegypti* larvae (16 larvae per group) in 48 well plates in 1.0 ml water. Larval mortality was recorded 24 h later. Controls were fed protein extract from cells that were not transformed. The results are expressed as averages of 4 or 8 determinations  $\pm$  S.E.M.

**Table 4. Feeding of Heat Treated and Untreated *P. pastoris* Cells Expressing *gst-cry11Aa-tmfa***

<i>P. pastoris</i> KM71H AOX1: <i>gst-cry11Aa-tmfa</i> cells were induced for 96 h and fed to mosquito larvae	LT <sub>50</sub> (days)	Confidence Limit (95%) (days)	LT <sub>90</sub> (days)	Confidence Limit (95%) (days)
a. Untreated	4.62	3.655-5.477	8.27	6.853-11.461
b. Heat inactivated at 50° C for 3 h	5.88	4.822-6.896	12.07	9.705-18.095

Cells were induced with methanol for 96 h, and 3 groups of 8 larvae were fed individually in 48 well plates for 12 days. The recombinant cells were untreated or heat inactivated at 50°C for 3 h. The mortality of each group was recorded at 24 h intervals for 12 days, and the results were subjected to probit analysis. Each experiment was repeated 3 times. LT<sub>50</sub>=Lethal time required to kill 50% of the larvae. LT<sub>90</sub>= Lethal time required to kill 90% of the larvae.

rapidly degrades the ingested toxin [37]. The proteases that are responsible for the inactivation of Cry1C are inhibited by trypsin-specific inhibitor such as PMSF. Thus, lowering the level of trypsin-like enzymes in the larval gut by TMOF might protect extensive degradation and inactivation of *Bti* toxins. Synergy is thus anticipated between TMOF and *Bti* enhancing larval killing at lower  $\delta$ -endotoxin concentrations. Thus, constructing genes encoding chimeric proteins that use trypsin to release TMOF and simultaneously activate the  $\delta$ -endotoxins is an advantage, and our report confirms this hypothesis.

We report here that starved *Ae. aegypti* larvae are 6-35 fold more sensitive to *Bti* toxins than well-fed larvae (Table 1), and starved 1<sup>st</sup> instar larvae are 10-fold more sensitive than 3<sup>rd</sup> instars. Since TMOF is more effective against 1<sup>st</sup> and 2<sup>nd</sup> 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.

The high toxicity of recombinant *E. coli* expressing *cry4Aa*, *cry11Aa* and *cyt1Aa* [27] to 1<sup>st</sup> instar *Ae. aegypti* larvae was dramatically reduced in the presence of *P. pastoris* as a nutritious food source (Table 2). Mortality was restored (about 95%) upon replacing it with recombinant *P. pastoris* producing TMOF (75-249 nM), which was not toxic alone (Table 2). These results confirm our hypothesis that TMOF and *Bti* are synergistic against larvae of *Ae. aegypti* in organic rich environment. TMOF enhances the toxicity of  $\delta$ -endotoxins by inhibiting trypsin biosynthesis, causing anorexia, starvation and death. These results confirm earlier observation that ingested nutritious particles reduce the susceptibility of insect larvae to  $\delta$ -endotoxins [38], indicating that other mechanisms (e.g. blocking of toxins receptor sites) may also play an important role. This observation explains why *Bti* is not effective against mosquito larvae in sewage treatment ponds. TMOF, on the other hand, traverses the gut epithelial cells and binds to its gut receptors at the

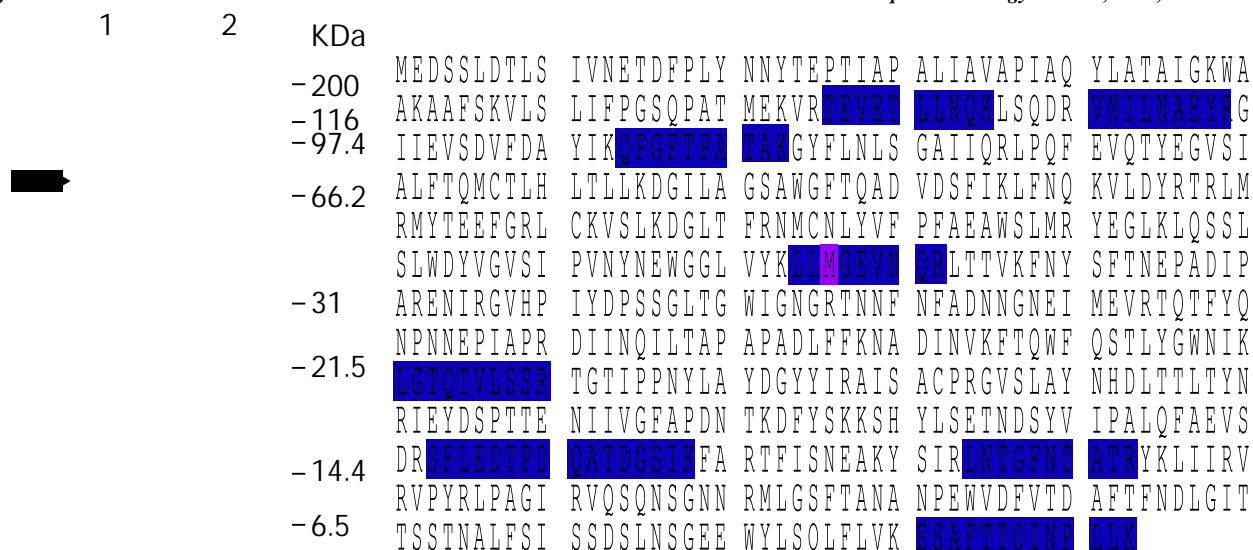
hemolymph side [6, 7, 34, 35]. Since TMOF and *Bti* have different mechanisms of action, increased resistance in exposed mosquito population against their combination is not likely. Although *Bti* and TMOF act like synergists in the laboratory, they must be tested under stringent field conditions for future larval control applications.

When *cry4Aa-tmfa* was cloned and expressed in *P. pastoris* KM71 cells without codon-optimization [28], the activity of the construct was poor. Codon optimized chimeric *cry11Aa-tmfa* and *gst-cry11Aa-tmfa* (Fig. 2) expressed in *P. pastoris* cells was effective against *Ae. aegypti* larvae (Figs. 3 and 4). Fusing GST and SUMO (small ubiquitin-related modifier) to proteins enhances their activity in bacteria and yeasts by increasing solubility in the cytoplasm [39]. Indeed, GST enhanced the heat stability of Cry11Aa-TMOF (Table 4), allowing heat inactivation of the yeast cells in future field applications without endangering the environment.

The GST-Cry11Aa recombinant protein produced in *Saccharomyces cerevisiae* without using codon optimized *gst-cry11Aa* was purified by affinity chromatography on glutathione Sepharose column [40]; the purified protein and the cell free crude extract were biologically active against 3<sup>rd</sup> instar *Ae. aegypti* larvae, but it was not reported whether the intact cells were also active. The cell free extract (5.69  $\mu$ g) of our *P. pastoris* clone expressing the chimeric *gst-cry11Aa-tmfa* was also biologically active, killing 40% of the early 4<sup>th</sup> instar *Ae. aegypti* larvae in 24 h (Table 3).

Ogunjimi *et al.* [41] cloned and expressed *cry2* in *P. pastoris* using pPICZ $\alpha$ B but did not report if the secreted protein was biologically active. A recombinant protein of 70 kDa was identified by SDS PAGE, but no mass spectrometry or N terminus analyses were reported that identified the putative protein as Cry2. Mass spectrometry analysis of Cry11Aa-TMOF that was purified from *P. pastoris* identified Cry11Aa but not TMOF that is fused to it with a trypsin cleavage site (IEGR) (Figs. 2 and 5). PCR-analysis of the recombinant cell DNA prior to the fermentation found a





**Fig. (5).** SDS-PAGE of Ni-NTA eluted proteins and mass spectra identification of Cry11Aa. *P. pastoris cry11Aa-tmfa* cells were fermented in the presence of methanol (0.5%) for 96 h, cells extracted with Y-PER and 8 M urea and proteins purified by affinity chromatography. Eluted proteins concentrated, separated by SDS-PAGE and stained with coomassie blue. A band at  $M_r$  72 kDa (lane 1, arrow) was identified, cut and incubated with trypsin and the released peptides were assayed by mass spectrometry. Sequences that were identified by mass spectrometry are stained yellow, except for M which is stained green. Lane 2, molecular weight standards.

full length gene (*cry11Aa-tmfa*). There are two ways to explain why TMOF was not detected by mass spectrometry: (a) the peptide was not cleaved in sufficient quantities during the tryptic digestion step, and (b) TMOF had been cleaved during the fermentation by yeast proteases releasing the hormone from the fusion protein into the cell cytoplasm. Because the His<sub>6</sub> tag is fused to Cry11Aa, the cleaved TMOF lacks it and thus did not bind to the Ni-NTA column and could not be detected. Our recombinant proteins that are produced inside *P. pastoris* cells are protected by the yeast's cell wall from sunlight and bacterial degradation, and are more palatable to mosquito larvae [10].

Photosynthetic cyanobacterial species are excellent candidates for toxin delivery [16, 20]. They are ubiquitous, float in the upper water layer and resist adverse environmental conditions. In addition, they are used as natural food sources for mosquito larvae [42, 43] and can be cultured on a large scale [44] and genetically altered [17-20, 45-48]. Therefore, co-expressing *cry*, *cyt* and *tmfa* in *Anabaena* PCC 7120 should have a potential advantage.

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