Received: July 30, 2007
Accepted: December 5, 2007
Abstract published online: December 7, 2007
Full paper published online: May 31, 2008

J. Venom. Anim. Toxins incl. Trop. Dis.
V.14, n.2, p.274-293, 2008.
Original paper.
ISSN 1678-9199.

HETEROLOGOUS EXPRESSION OF AN INSECTICIDAL GENE FROM THE ARMED SPIDER (*Phoneutria nigriventer*)

FIGUEIREDO J. E. F. (1), KALAPOTHAKIS E. (2), GOMEZ M. V. (2), BRESSAN W. (1)

(1) Laboratory of Molecular Biology, Embrapa Maize and Sorghum, Sete Lagoas, Minas Gerais State, Brazil; (2) Department of Pharmacology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais State, Brazil.

ABSTRACT: Insect-pests are global problems that cause severe damage to crop plants, and their control is commonly based on chemical insecticides. However, negative effects of pesticides on the environment and human health emphasize the necessity to develop alternative methods for insect-pest control. In the present study, a gene coding for the insecticidal peptide TX4(6-1) of the Brazilian armed spider (*Phoneutria nigriventer*) was cloned in fusion with maltose binding protein (MBP) and expressed in *Escherichia coli*. The affinity purified protein MBP-GlyTX4 was cleaved with the Xa factor and used for a bioassay against *Spodoptera frugiperda* and rabbit immunization. Five micrograms GlyTX4 protein injected into the hemocoel of larvae and abdominal cavity of adults produced trembling and uncoordinated movements immediately after injection and all adult insects died after 12h. After two days, larvae became paralyzed and the epidermal color changed to dark brown. Furthermore, the development stage was prolonged for two weeks. Alternatively, slices of maize leaves were imbibed with 15 micrograms of the recombinant protein cleaved with the Xa factor and used as diet for larvae. In this experiment, all larvae died in about 30 minutes. Polyclonal antibodies anti-MBP-GlyTX4 were effective for recognizing MBP and GlyTX4 in whole cell extract from *E. coli* expressing the recombinant protein.

KEY WORDS: bioinsecticide, molecular cloning, heterologous expression, *Escherichia coli*, *Spodoptera frugiperda.*

CONFLICTS OF INTEREST: There is no conflict.

CORRESPONDENCE TO:
JOSÉ EDSON FONTES FIGUEIREDO, Laboratório de Bioquímica Molecular, Embrapa Milho e Sorgo, Rodovia MG 424, Km 65, Caixa Postal 151, 35 701-970. Phone: 55 31 3779 1169. Fax: 55 31 3779 1088. Email: jeff@cnpms.embrapa.br.

INTRODUCTION

Arthropod pest species destroy about a quarter of the world's annual crop production and transmit an impressive array of human and veterinary pathogens (3, 18, 34). Maize insect-pests that attack different stages of the crop are serious problems in tropical areas. About one billion dollars are lost annually in the field due to pest attack, even with chemical control. Since the introduction of DDT in the 1940s, insect pests have been controlled almost exclusively by a limited number of broad-spectrum chemical insecticides that have led to the development of resistant populations in several important pest groups (3). The abusive and indiscriminate use of chemical insecticides is responsible for environmental and ecological damage to natural enemies and the death of millions of birds and fishes along with the insect-pests (18, 29). In addition, there is strong epidemiological (17, 27) and experimental (1, 42) evidence linking the occurrence of cancer, Parkinson's disease and others neurological disorders to pesticide exposure. Thus, it is important at this juncture to identify and characterize novel compounds with insecticidal activity. The development of new strategies for pest control by using organisms and/or natural substances to minimize biological and environmental damage caused by chemical insecticides could be an important alternative for reducing agricultural costs (29, 41).

Spider venom contains a vast array of biologically active substances, some of which are toxins. They are supposed to be rich source of insecticidal compounds since the primary action of spider venom is to kill or paralyze arthropod prey by targeting the nervous system of these organisms. The specificity of some spider toxin acting only in insects has an enormous potential for application as bioinsecticides. The x-ACTX-Hv1a toxin (Hvt) found in the venom of the Australian funnel web spider (*Hadronyche versuta*) is an insect-specific calcium-channel antagonist (33). The peptide is toxic to a range of agriculturally important arthropods in the Coleoptera, Lepidoptera and Diptera orders and has been reported to have no effects on a number of mammals (33). Recently, active recombinant spider toxins have been cloned and expressed in prokaryotic (14, 26) and eukaryotic systems (14, 15), and transgenic plants expressing spider insecticidal peptides are resistant to insect attack (26).

Spider venom consists of a complex mixture of substances containing a variety of toxic components. Polypeptides with insecticidal activity isolated from the venom glands of different spider species display spatial structure homology and interact with

ion channels of the excitable membrane, affecting its functioning (5, 16, 19, 32, 36, 43).

Numerous low molecular weight polypeptides with neurotoxic activity were identified in extracts from the venom glands of the South American armed spider *Phoneutria nigriventer* (13, 16, 35). The chromatographic fraction TX4(6-1) of *P. nigriventer* venom is specific against insects with no toxic effect to avian and mammalian species (35). Tx4(6-1) toxin was highly active against house flies, producing neurotoxic effects at the low dose of 0.02ng/mg and against cockroaches, at doses ranging from 0.5 to 2.5ng/mg (35). Bioassay with Tx4(6-1) polypeptide against important maize crop insect-pests could lead to new technological insights for insect-resistant plants in agriculture. However, besides the difficulties in rearing a great number of spiders to isolate a high number of venom glands, the amount of Tx4(6-1) polypeptide required for bioassays is very expensive due to the difficulties in obtaining it with the currently available chromatographic methods. Molecular cloning techniques using heterologous systems for the expression of high amounts of recombinant polypeptide coupled with affinity chromatographic methods could minimize those difficulties.

Many cDNA clones encoding *P. nigriventer* neuropeptides were isolated by screening a venom gland cDNA library (35). Computer analysis and experimental data revealed that a cDNA clone [Tx4(6-1)] encodes an inactive polypeptide with 82 amino acids with 16aa residues at N-terminus corresponding to the endoplasmic reticulum (ER) signal peptide. The remaining polypeptide with 66aa residues is secreted into the cavity of venom glands and cleaved by an unknown protease giving rise to the active insecticidal polypeptide that is 48aa in length and stabilized by disulfide bridges. In the present study, a DNA sequence encoding for the active TX4(6-1) insecticidal peptide of *P. nigriventer* was cloned in fusion with MBP, expressed in *Escherichia coli*. The affinity purified protein was used for rabbit immunization and bioassay against S*podoptera frugiperda*, the most important insect-pest of the maize crop in the tropics.

MATERIALS AND METHODS

A cDNA clone [TX4(6-1)] encoding a precursor toxin with insecticidal activity was previously isolated from *P. nigriventer* venom gland cDNA library (35). The TX4(6-1) clone was used as template to amplify the coding region for the active peptide by

Polymerase Chain Reaction (PCR). Two primers flanking the DNA region of interest were used: Forward, 5'-GAAGAATCGCCCGGGTGCGGCG-3' and Reverse, 5'-CGAGGTATTCACGGATCCA CC-3' (Figure 1). PCR reaction with a total volume of 25μl contained: 100-200ng of DNA template, 20μM of each primer, 25μM dNTP, 2.5mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (*Phoneutria*, Belo Horizonte, MG, Brazil). Amplification was performed in a Thermal Cycler (PTC-100, MJ Research, Inc.) under the following conditions: a 1min cycle at 94^{o}C, 40 cycles of 1min at 94^{o}C, 1min at 50^{o}C and 2min at 72^{o}C. After the last cycle, a single step at a temperature of 72^{o}C was performed to ensure that DNA was fully extended. Five microliters of the PCR reaction was loaded on 0.8% agarose gel and the amplified product was visualized under UV light. Afterwards, the 144bp long DNA fragment was double digested with *Sma* I and *Bam*H I restriction enzymes for 2h at 30^{o}C and 37^{o}C, respectively. The digested DNA was resolved in 0.8% agarose gel, purified with GeneClean II kit (BIO 101, Irvine, CA, USA) and cloned in pUC 18 vector. Ligation reaction and transformation methods were according to Sambrook and co-workers (38). Two positive clones were sequenced at least twice (forward and reverse) in order to check the fidelity of the Taq DNA polymerase activity and the open reading frame (ORF) integrity. Afterwards, the coding sequence for the active Tx4(6-1) peptide was released from pUC18 by *Sma* I and *Bam*H I restriction enzymes, purified from 0.8% agarose gel and cloned in the polylinker region of the pMAL-2 (p2) expression vector digested with *Xmn* I and *Bam*H I. During this procedure, a GGG codon for glycine (G) was introduced in the TX4(6-1) sequence coding for the GlyTX4 active peptide (Figure 1) and fused with maltose binding protein (MBP) gene. The M15 *E. coli* strain was cotransformed with the recombinant plasmid pMAL-MBP-GlyTX4 and the pREP plasmid. Positive clones harboring the MBP-GlyTX4 gene were checked by sequencing using the pMAL-2 reverse primer (New England Biolabs, Ipswich, MA, USA). The schematic representation of molecular cloning and the expression of the MBP-GlyTX4 recombinant protein in *E. coli* are shown in Figure 2. The MBP-GlyTX4 recombinant protein was induced and purified using pMAL-2 Protein and Purification System (New England Biolabs, Ipswich, MA, USA) according to the method described in the procedure manual accompanying the reagents. Briefly, an aliquot of fresh overnight culture of cotransformed *E. coli* was transferred to an erlenmeyer flask containing 50ml Luria-Bertani (LB) broth medium (Difco Laboratories, Detroit, MI, USA) supplemented with 2% maltose, 100μg/ml ampicillin

and 25μg/ml kanamycin (Sigma, St Louis, MO, USA). The bacterial culture was incubated at 37°C by shaking at 300rpm until the culture reached OD_{600}=0.6. At this stage, the expression of the recombinant protein was induced by IPTG (isopropylthiogalactoside) at the final concentration of 0.3mM. After 4h, the culture was harvested by centrifugation at 3000rpm for 10min at 4°C and the supernatant was discarded. The pellet containing induced cells was suspended in 10ml column buffer (2mM Tris-HCl, pH 7.4, 200mM NaCl, 1mM EDTA, 1mM sodium azide, 10mM β-mercaptoethanol) and incubated at -20°C overnight. The lysed cells were placed in ice-water bath, sonicated 5 times with short pulses of 15s each and centrifuged at 10,000rpm for 30min. The supernatant was recovered and diluted 5 times with column buffer and chromatographed in amylose column. Afterwards, the column was washed with five volumes of the column buffer and MBP-GlyTX4 fusion protein was eluted with 10mM maltose with a flow rate adjusted to 0.8ml/min in fractions of 3ml. The identity of the recombinant protein was confirmed by western blot with anti-MBP antibody and its purity was confirmed by a 12% sodium dodecyl sufate (SDS)-polyacrylamide gel electrophoresis stained with Coomassie Blue R250 (BIO-RAD Laboratories, Hercules, CA, USA) and by the silver staining method. The purified MBP-GlyTX4 was used for rabbit immunization according to Sambrook and co-workers (38).

MBP-GlyTX4 fusion protein was cleaved with the Xa factor and used for bioassay against larvae at the fourth instar stage and adults of *S. frugiperda*. The control consisted of 10 larvae and 10 adults inoculated with 10μl of autoclaved distillated water or 10μl of column buffer or 10μl of sample buffer containing 15μg of MBP protein plus the Xa factor. All experiments were repeated at least three times.

The insecticidal activity of the recombinant protein was evaluated by injecting 5μg of the purified MBP-GlyTX4 cleaved with the Xa factor into the hemocoel of larvae and the abdominal cavity of adult insects. The median lethal dose (LD_{50}) and completely lethal dose (LD_{100}) of the recombinant protein injected into adult insects were 2.565μg/kg^{-1} and 4.923μg/kg^{-1} of body weight, respectively, as estimated by “Probit” analysis using the SAS computer program. The estimated dose-response equation was Y=2,313+6,567X, where Y=“Probit” value that could be converted to percentage of mortality and X is the logarithm of the toxin doses expressed by μg/ml^{-1}. The Chi-square test of significance was χ^2=3,134 with p=0.961. In another experiment,

3X0.5cm-long pieces of maize leaves were immersed for five minutes in a solution containing 15μg cleaved MBP-GlyTX4 protein and supplied as the single food source for the larvae. The median lethal concentration (LC_{50}) and LC_{100} of the recombinant protein used for leaf immersion were 9.524μg/cm^2 and 14.268μg/cm^2 of leaf, respectively. The "Probit" equation was Y=-1.0227+6.153X. The Chi-square test (χ^2) value was highly significant (18,739) and p=0.998.

RESULTS AND DISCUSSION

The complete TX4(6-1) cDNA clone is 401bp in length and 25bp corresponds to the 5'untranslated region, 249bp corresponds to the ORF and the stop codon, and the 3'untranslated region (UTR) that is 127bp long (Figure 1). The ORF is divided into two parts: the sequence encoding the endoplasmic reticulum signal peptide with 48bp and the inactive peptide with 198bp and 144bp corresponds to the sequence encoding the TX4(6-1) active peptide. The small length of DNA encoding the TX4(6-1) active peptide and the absence of restriction sites immediately adjacent to the first codon for functional toxin in TX4(6-1) cDNA are cloning restrictive' (Figure 1). Furthermore, designing a restriction site in the forward primer may prevent the introduction of codons for highly reactive amino acids in the recombinant DNA to minimize the risk of structural and functional changes in the recombinant molecule. In the present study, a *Sma* I restriction site created in the forward primer was the best choice. Within this procedure, after digestion of the amplified DNA with *Sma* I, a GGG codon for glycine was introduced just upstream to the first original codon of TX4(6-1) active peptide (Figure 1). Additionally, the *Sma* I restriction site is the first 5' upstream site for cloning in pMAL-2 vector and it is located in the cleavage site of the Xa factor. Besides adding a non-essential and neutral protein-forming amino acid without a center of chirality to the recombinant neurotoxin, this procedure avoided the incorporation of any MBP amino acid to the GlyTX4 peptide after cleavage with the Xa factor. This strategy was important to minimize the possible mutational effect of MBP amino acids incorporated in the recombinant neuropeptide. After cloning in pUC 18, the addition of a GGG codon for glycine at the 5' end of the DNA encoding for TX4(6-1) active peptide was confirmed by DNA sequencing (data not shown). The insert encoding the mutated TX4 insecticidal peptide (GlyTX4) was excised from pUC 18 with the *Sma* I and *Bam*H I restriction enzymes and cloned in pMAL-2 expression

vector previously digested with *Xmn* I and *Bam*H I. This procedure generated the recombinant MBP-GlyTX4 gene, as confirmed by DNA sequencing analysis using the pMAL-2 reverse primer (New England Biolabs, Ipswich, MA, USA).

The expression of recombinant proteins in pMAL-2 vector is regulated by *lac* operon. Although the *lac* promoter is well repressed in the absence of inducers, detectable basal activity always occurs (20). Considering the complete absence of previous knowledge about the biochemical behavior of the TX4(6-1) protein expressed in heterologous systems, an additional control over gene expression is desirable to avoid possible premature host cell death due to toxic effects of the GlyTX4 recombinant protein. pRep plasmid encoding a stronger *lac* operon repressor was used to prevent undesired expression of recombinant protein before induction. In the present study, the expression of recombinant protein was not observed before the induction with IPTG when both the recombinant pMALMBP-GlyTX4 and pRep plasmids were used together for *E. coli* transformation (Figures 2 and 3).

Spider toxins have a handful of the strictly conserved cysteines that direct the three-dimensional fold of proteins (40). DNA analysis of the TX4(6-1) cDNA clone predicted that mature neurotoxic peptide displays ten cystein residues, which means five potential disulfide bonds formation (Figure 1). Disulfide bonds are a stable part of the final folded structure of proteins and contribute to the folding pathway and stability of their native state (30). Disulfide bonds are typical post-translational modifications of secretory proteins. In bacteria, disulfide bond formation occurs in the oxidizing environment of the periplasm and is catalyzed by redox enzymes of the Dsb family (24, 37). The p2 version of the pMAL-2 vector, which secretes the target protein to the periplasm was used due to the high number of cystein residues in the small TX4 active peptide (Figure 1).

A high amount of the recombinant protein was obtained after cell induction with IPTG (Figure 3). A 200ml amylose column was completely saturated with 50ml culture extract, obtaining 320μg of the total recombinant protein per ml culture as estimated by the Bradford protein assay (2). The high level of expression of the fusion protein (more than 50% of the total soluble protein) was achieved without apparent toxic effects on bacterial growth. In another study (26), the expression of ω-ACTX-Hv1a toxin (Hvt), a component of the venom of the Australian funnel web spider (*Hadronyche versuta*) in an *E. coli* system, produced more than 30% of the total soluble protein. Both the present results with recombinant GlyTX4 peptide and the

results obtained by Khan and co-workers (26) with ω-ACTX-Hv1a toxin (Hvt) indicated that *E. coli* is a very efficient system for expressing functional spider recombinant peptide carrying high numbers of sulphydryl residues.

Although the native MBP and the recombinant MBP-GlyTX4 have different molecular mass, as indicated by computer analysis, it was difficult to visualize differences between both proteins in a 12% SDS-PAGE gel (Figure 3a). Molecular masses of MBP cloned in pMAL-2 and MBP-GlyTX4 are 50831.22Da and 48760.08Da, respectively. However, differences in length between both proteins became apparent by using sensitive methods such as the western blot (Figure 3b).

Heterologous expression of insecticidal toxins in plants, to protect them from herbivorous insects attack has been proven as an effective means of reducing farmer's reliance on environmentally harmful chemical alternatives (26). Recently, the transgenic expression of ω-ACTX-Hv1a toxin (Hvt) in tobacco effectively protected the plants from *Helicoverpa armigera* and *Spodoptera littoralis* larvae, with 100% mortality within 48h (26). We have investigated the potential usefulness of expressing the Brazilian armed spider venom toxin TX4(6-1) in maize plants to provide protection against *Spodoptera frugiperda*. Maize transgenic plants expressing the TX4(6-1) active peptide targeted to the cytoplasm showed abnormal development and all plants died before the adult phase (Figueiredo and Carvalho, unpublished results). Whether maize plant deleterious phenotypes were due to toxic effects of the TX4(6-1) transgene itself or to cellular compartmentalization of TX4(6-1) to cytoplasm needs to be investigated. In the present study, polyclonal antibodies produced against MBP-GlyTX4 were effective for recognizing MBP-GlyTX4 in whole cell *E. coli* extract as well as potential MBP and GlyTX4 proteins cleaved with the Xa factor (Figure 4). Anti-MBP-GlyTX4 polyclonal antibodies will be useful to immunocytochemical assays to evaluate TX4(6-1) bioinsecticide targeted to different plant subcellular compartments.

Insects are the primary source of food for most spider diets. Spiders inject venom into the body of a captured insect to paralyze it. To some extent, injections of toxin into the abdominal cavity of insects simulate the injection of spider venom into insects. In the present study, the bioassay against *S. frugiperda* was performed with five micrograms (LD_{100} for adult insects) of GlyTX4 recombinant protein injected into the hemocoel of larvae and abdominal cavity of adult insects. The larvae ceased feeding and showed the behavioral symptoms typical of this group of neurotoxins. Both

larvae and adults showed trembling and uncoordinated movements immediately after injection. All adult insects died within 12h after application whereas larvae showed diminished movements, changes in epidermal color to dark brown and the developmental stage was prolonged for 2 weeks (Table 1). The difference in toxicity of recombinant GlyTX4 between larvae and adults was not clear. However, two points could be considered. First, neurotoxic activity of spider toxins is due to the interaction of the venom components with cell receptors, in particular, ion channels (12). Since adult insects are the natural spider's prey, spider toxin may interact with more affinity with some specific receptor present in adult insect hemolymph. A second consideration is based on the fact that adult insects are more sensitive than larvae. In this case, GlyTX4 could be more toxic to adult insects.

Symptoms induced by the recombinant GlyTX4 toxin in *S. frugiperda* were identical to those described for the native TX4(6-1) venom peptide isolated from *P. nigriventer* (13, 35) consisting of a lack of coordination, disorientation and uncontrolled movement. The results obtained with GlyTX4 expressed in *E. coli* and the studies with ω-ACTX-Hv1a toxin (Hvt) expressed in transgenic plants (26) showed that the mode of action of the heterologously expressed spider peptides are similar. The effects of spider toxin against insects were first observed by injecting *Heliothis virescens* (Lepidoptera:Noctuidae) larvae with three polypeptide toxins (SFl1) purified from *Segestria florentina* venom glands (28). Those polypeptides had no toxic effects on intravenously injected mice (28). Likewise, the native TX4(6-1) of *P. nigriventer* is specific against insects with no toxic effect to avian and mammalian species (13, 35).

Insecticidal spider venom toxin (*Segestria florentina* toxin 1, SFI1) fused to snowdrop lectin is toxic to the peach-potato aphid *Myzus persicae* (Hemiptera:Aphididae) and the rice brown planthopper *Nilaparvata lugens* (Hemiptera:Delphacidae) by incorporation into artificial diets (10). In the present study, slices of maize leaves immersed in 15μg recombinant protein cleaved with the Xa factor were supplied as diet for larvae. All the larvae were knocked down immediately and died within 30min (Table 1). This result is important in considering that the insecticidal recombinant protein in transgenic plants will be expressed and accumulated in maize leaves, the primary source of food for *S. frugiperda*. Biological toxicity assays using the recombinant protein against *S. frugiperda* demonstrated that the GlyTX4 peptide expressed in *E. coli* had toxic effect when injected into the larva hemocoel (larval stage prolonged) and when injected into the adult abdominal cavity (death). Similarly,

GlyTX4 showed insecticidal activity when supplied as diet to larvae (death). The effects of GlyTX4 injected into the larva hemocoel and supplied as diet were very different. Difference in pH values between the larvae's midgut and hemocoel as well as specific proteases present in the hemolymph could explain the above results. Protein unfolding and denaturation at different pH is quite a general phenomenon (38). Changes in pH or ionic strength can bring about changes in protein conformation, which in turn can affect the toxin interaction with its receptor and affect its activity (23). Many *Bacillus thuringiensis* toxins drastically increased their surface hydrophobicity, which correlated with increased binding at low pH (4). Similar changes in surface hydrophobicity were observed in many unrelated proteins (19, 34). In *Manduca sexta* (Lepidoptera), pH on the lumen side is 8.18 and hemolymph pH is 6.7 (38). Lumen pH values of approximately 11.0 are common in other species (8). Thus, the nearly neutral pH in the hemocoel of *S. frugiperda* could be responsible for conformational changes in the GlyTx4 protein, inactivating thus the toxin. Experiments with GlyTx4 toxin to measure its activity in different pH ranges are necessary to confirm this hypothesis. Second, insect hemolymph is a dynamic environment responsible for insect defense against injury, parasites and toxins. The proteinaceous component of the insect hemolymph is complex, both in structure and function (25). Different proteases and protease inhibitors have been identified in the insect hemolymph (7). Specific hemolymph protease activity may be responsible for partial inactivation of the GlyTx4in larvae injected with the toxin. Physiological and biochemical studies as well as immunocytochemical assays with *S. frugiperda* ligand and GlyTX4 using antibodies against the MBP-GlyTX4 recombinant protein are necessary to elucidate the present result.

Oxidative protein folding is a composite process by which a reduced, unfolded protein acquires both its native disulfide bonds and native structure (31). Failure to form proper disulfide bonds, or their slow formation in the cell, is likely to lead to protein aggregation and degradation by proteases. In eukaryotic cells, the lumen of the endoplasmic reticulum (ER) is a compartment specialized for protein folding. Protein folding and isomerization in this context is often associated with the formation of native disulphide bonds, and this is facilitated by the enzyme protein disulphide isomerase (PDI) and Erro1 (6). In gram-negative bacteria, disulfide bonds are formed in the oxidizing environment of the periplasm (22, 24). Disulfide bonds can be formed spontaneously by molecular oxygen *in vitro*, but the rate of this spontaneous

formation is much slower than the rate *in vivo*. Moreover, the number of possible disulfide cross-links increases by around one order of magnitude with each additional cysteine pair. This means that a protein with four pairs of cysteine residues has less then a 1% chance of attaining the correct four disulfides by random oxidation (37). Indeed, *in vivo* oxidative protein folding is catalyzed by a variety of thioldisulfide oxidoreductases and requires two enzymatic activities: disulfide bond formation and isomerization of non-native disulfide bonds. In the periplasm of *Escherichia coli*, Dsb proteins catalyze disulfide bond formation, isomerization and reduction. Toxic effects of the eukaryotic GlyTX4 recombinant peptide to *S. frugiperda* indicated that GlyTX4, similar to ω-ACTX-Hvla toxin (26), retains its biological activity when expressed in the *E. coli* prokaryotic system suggesting that disulfide bonds in eukaryotic proteins were properly made in the reducing prokaryotic *E. coli* periplasmic space, and that GlyTX4 was properly folded and reached its native state.

Although the toxic symptoms observed for GlyTX4 and the native TX4(6-1) were very similar, both proteins could be structurally different due to post-translational modifications and differences inherent to post-translational machinery between both systems (eukaryote *versus* prokaryote). A high order of complexity can be added after a gene has been translated into the corresponding protein (9). Post-translational modifications can endow proteins with properties that are very different from those that are predicted by the encoding genes. Post-translational modification is a common phenomenon in toxins (32). Changes in the three-dimensional toxin structure provided by these modifications result in optimal binding specificity for receptor isoforms and subtypes, which is particularly important for successful prey hunting (23). The dynamic complexity of post-translational modifications in eukaryotes ranges from the widespread (such as glycosylation, phosphorylation, ubiquitination, and methylation) to the obscure (such as glutathionylation, hydroxylation, sulfation, transglutamination, and epimerization) and their effects often fundamentally alter protein function (8, 9). In several cases, modifications once thought to be restricted to Eucarya were discovered in prokaryotic cells. The glycosylation of the *Halobacterium salinarum* S-layer glycoprotein represents one such example (11). Although bacterial proteins can undergo post-translational modifications, the cellular machinery as well as the mode and frequency in which they are observed are very different (11). For example, post-translational modification of proteins by covalent attachment of carbohydrate moieties is common among

eukaryotes but, by comparison, relatively rare in bacteria (21). Spider venom peptides are translated mostly as prepropeptides that undergo post-translational modification to yield the mature toxin (39). Although post-translational modifications could be expected to occur in the native TX4(6-1) protein, structural variation in the TX4(6-1) spider venom toxins is yet to be established. In the present study, the bioinsecticidal activity of the recombinant GlyTX4 protein indicates that processing in *E. coli* was effective to convert the precursor GlyTX4 to an active toxin. In addition to the fact that the recombinant GlyTX4 protein was expressed in a very different biological environment (prokaryote), bacterial and eukaryotic machinery for post-translational modifications differs in many aspects. Thus, it is possible that GlyTX4 and the native TX4(6-1) have different structural variations. Comparative analysis applying mass spectrometry (MS) and crystallography will be performed to elucidate the occurrence of post-translational modifications in both the native TX4(6-1) and the recombinant GlyTX4 toxin.

Thereafter, the insecticidal effect of recombinant GlyTX4 toxin from the armed spider (*Phoneutria nigriventer*) indicated that the *E. coli*/MalE system could be an efficient alternative system to express eukaryotic peptides, even those rich in cysteine residues. We conclude that the TX4(6-1) polypeptide from *P. nigriventrer* is an attractive and effective molecule for transgenic protection of plants from herbivorous insects which should be further evaluated for possible application in agriculture.

Figure 1. Partial sequence and deduced precursor polypeptide of the TX4(6-1) cDNA clone. The signal peptide with sixteen amino acid residues responsible for targeting the propolypeptide to endoplasmic reticulum is underlined and the shaded sequence corresponds to amino acid residues removed by proteases in the lumen of the venom glands (35). The first and the last amino acid residue in the native active TX4(6-1) peptide are indicated by numbers 1 and 48, respectively. The position of a glycine residue introduced in the active peptide sequence after cloning in pMAL-2 (*) and stop codon (***) are indicated. Shadowed regions in DNA indicate the position and sequence of synthetic primers used for amplification by PCR. Mutations creating recognition sites for *Sma* I (CCCGGG) and *Bam*H I (GGATCC) restriction enzymes are shown above the original cDNA sequence.

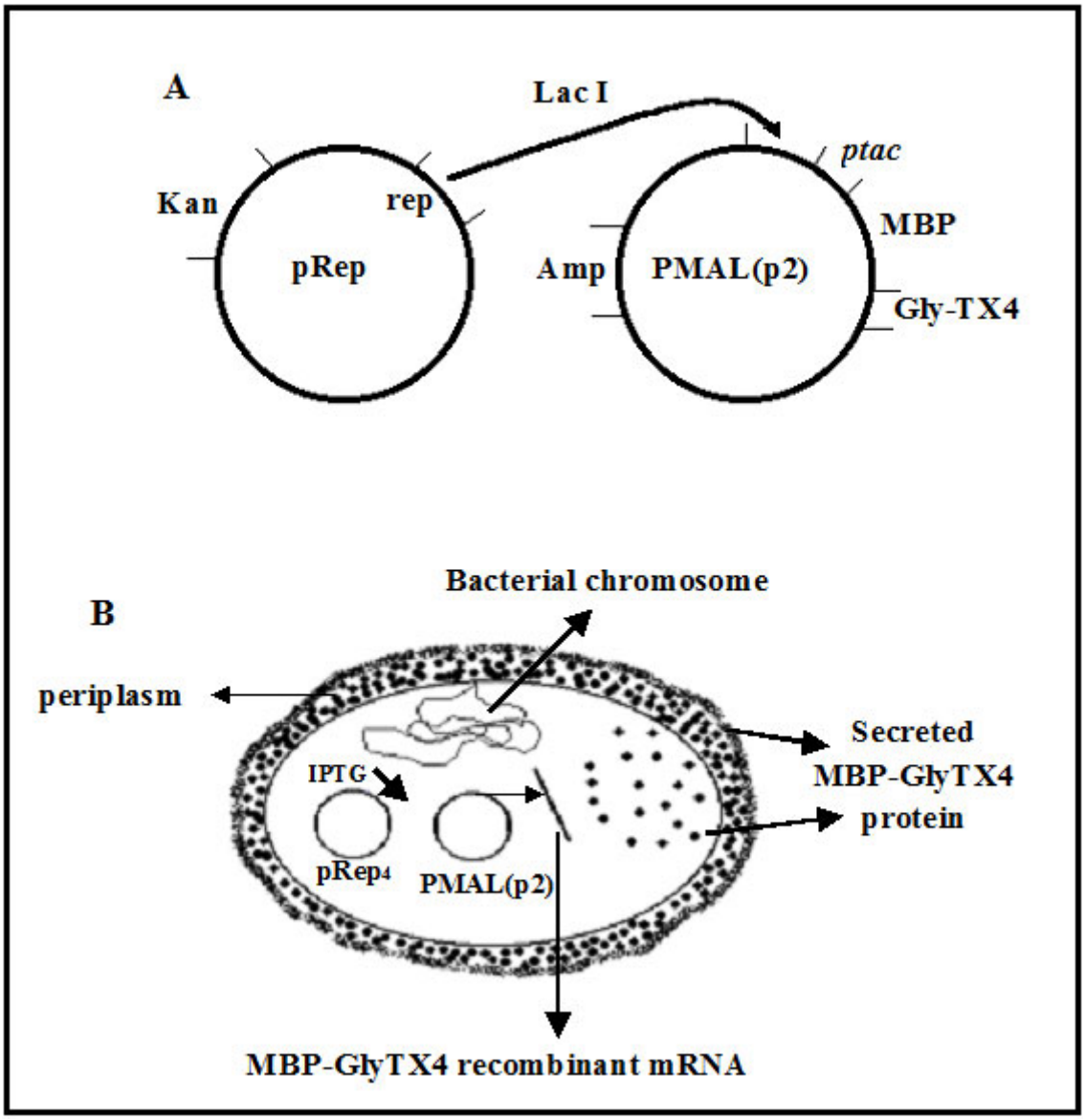

Figure 2. Diagram of molecular cloning (A) and expression (B) of the MBP-GlyTX4 fusion protein. The recombinant pMAL-2MBP-GlyTX4 and the *lac* repressor (pREP) plasmid were used for cotransformation of the *Escherichia coli* M15 strain. The recombinant MBP-GlyTX4 protein was induced by IPTG and targeted to bacterial periplasm.

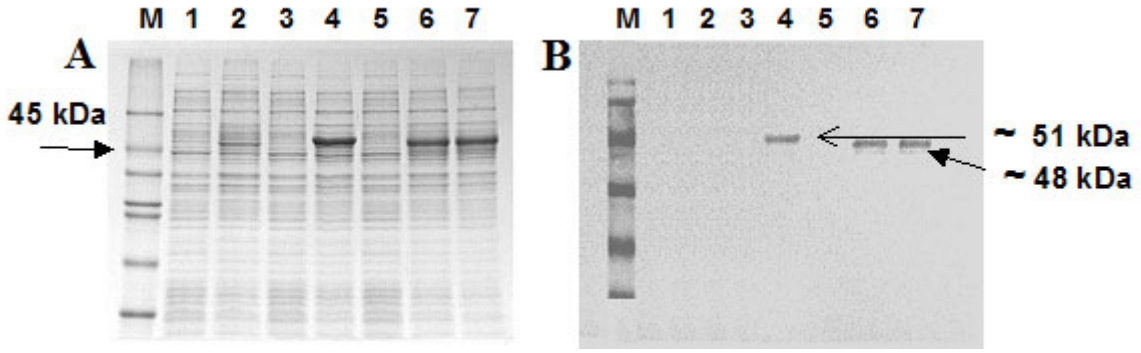

Figure 3. SDS-PAGE (A) and western blot (B) of whole cell extract of *Escherichia coli* non-assayed with anti-MBP antibody (New England Biolabs, Ipswich, MA, USA). Lines 1, 3 and 5 were loaded with non-induced whole cell extract of bacteria transformed with pREP, pMAL E and recombinant pMAL-2-MBP-GlyTX4, respectively. Lines 2 and 4 were loaded with pREP and pMAL-2 (p2) cultures induced with 10mM IPTG, respectively. Lines 6 and 7 are whole cell culture extracts harboring the recombinant pMAL-2-MBP-GlyTX4 after 4h and 8h of induction with 10mM IPTG, respectively. M = rainbow molecular weight marker (New England Biolabs, Ipswich, MA).

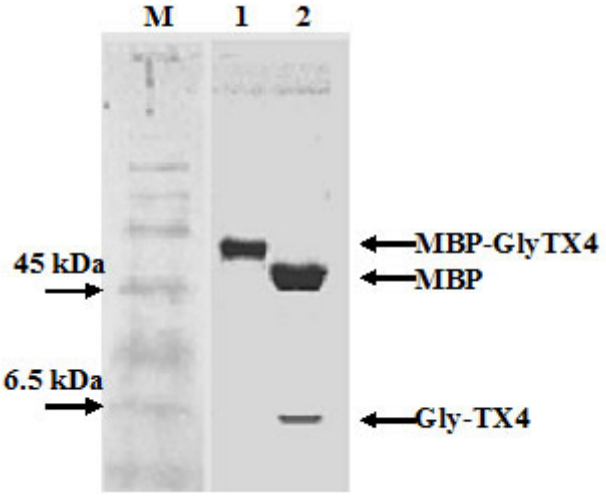

Figure 4. Western blot of the purified MBP-GlyTx4 recombinant protein assayed with rabbit polyclonal anti-MBP-GlyTX4 antibody. Line M: Ponceau-stained molecular weight marker (New England Biolabs, Ipswich, MA, USA); Line 1: Affinity purified recombinant MBP-GlyTX4 protein; Line 2: Affinity purified recombinant MBP-GlyTX4 protein cleaved with the Xa factor. The antibody was diluted 3000X.

Table 1. Bioassay with *Spodoptera frugiperda* using the affinity purified MBP-GlyTX4 recombinant protein cleaved with the Xa factor.

Treatments (applications)	**Stage**	**Doses**	**Individuals**	**Mortality (%)**	**Time (h) (approximately)**
Ventral	Larvae	5 μg	40	0	---
Ventral	Adults	5 μg	40	100	12
Diet (leaf)	Larvae	15 μg	40	100	1/2
Control	Larvae/Adults	---	70	0	---

--- No death was observed within two weeks (larvae injected with the recombinant protein) or within 30h (larvae fed with slices of maize leaves imbibed with the recombinant protein).

ACKNOWLEDGEMENTS

The authors thank CNPq/Pronex - National Council for Scientific and Technological Development/Support Program for Excellence Centers [Conselho Nacional de Desenvolvimento Científico e Tecnológico/Programa de Apoio a Núcleos de Excelência] and FINEP (Financiadora de Estudos e Projetos) for funding. We also thank Edna Patto Pacheco de Pinho and Osmar Santana de Souza for technical support. We are grateful to reviewers for providing valuable suggestions and discussions.

REFERENCES

1 BETARBET R., SHERER TB., MACKENZIE G., GARCIA-OSUNA M., PANOV AV., GREENAMYRE JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.*, 2000, 3, 1301-6.

2 BRADFORD MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem.*, 1976, 72, 248–54.

3 BROGDON WG., MCALLISTER JC. Insecticide resistance and vector control. *Emerg. Infect. Dis.*, 1998, 4, 605-13.

4 BUTKO P., COURNOYER M., PUSZTAI-CAREY M., SUREWICZ WK. Membrane interactions and surface hydrophobicity of *Bacillus thuringiensis* delta-endotoxin CryIC. *FEBS Lett*., 1994, 340, 89–92.

5 CATTERALL WA., CESTÈLE S., YAROV-YAROVOY V., YU FH., KONOKI K., SCHEUER T. Voltage-gated ion channels and gating modifier toxins. *Toxicon*, 2007, 49, 124-41.

6 CHAKRAVARTHI S., JESSOP CE., BULLEID NJ. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *Eur. Mol. Biol. Org. Rep.*, 2006, 7, 271–5.

7 CHERQUI A., CRUZ N., SIMÕES N. Purification and characterization of two serine protease inhibitors from the haemolymph of *Mythimna unipuncta*. *Insect Biochem. Mol. Biol*., 2001, 31, 761-9.

8 CRAIK DJ., DALY NL., WAINE C. The cystine knot motif in toxins and implications for drug design. *Toxicon*, 2001, 39, 43-60.

9 DAVIS BG. Mimicking posttranslational modifications of proteins. *Science*, 2004, 303, 480-2.

10 DOWN RE., FITCHES EC., WILES DP., CORTI P., BELL HA., GATEHOUSE JA., EDWARDS JP. Insecticidal spider venom toxin fused to snowdrop lectin is toxic to the peach-potato aphid, *Myzus persicae* (Hemiptera:Aphididae) and the rice brown planthopper, *Nilaparvata lugens* (Hemiptera:Delphacidae). *Pest. Manag. Sci.*, 2006, 62, 77-85.

11 EICHLER J., ADAMS MWW. Posttranslational protein modification in *Archaea*. *Microbiol. Mol. Biol. Rev*., 2005, 69, 393-425.

12 ESCOUBAS P., DIOCHOT S., CORZO G. Structure and pharmacology of spider venom neurotoxins. *Biochimie*, 2000, 82, 893–907.

13 FIGUEIREDO SG., LIMA-PEREZ GME., VALENTIM ADC., CORDEIRO MN., DINIZ CR., RICHARDSON M. Purification and amino acid sequence of the insecticidal neurotoxin TX4(6-1) from the venom of the “armed” spider *Phoneutria nigriventer* (Keys). *Toxicon*, 1995, 33, 83-93.

14 FITCHES E., AUDSLEY N., GATEHOUSE JA., EDWARDS MG. Fusion proteins containing neuropeptides as novel insect control agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. *Insect. Biochem. Mol. Biol.*, 2002, 32, 1653-61.

15 FITCHES E., EDWARDS MG., MEE C., GRISHIN E., GATEHOUSE AMR., EDWARDS JP., GATEHOUSE JA. Fusion proteins containing insect-specific toxins as pest control agents: snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. *J. Insect. Physiol*., 2004, 50, 61-71.

16 GOMEZ MV., KALAPOTHAKIS E., GUATIMOSIM C., PRADO MAM. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. *Cell. Mol. Neurobiol.*, 2002, 22, 579–88.

17 GORELL JM., JOHNSON CC., RYBICKI BA., PETERSON EL., RICHARDSON RJ. The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. *Neurology*, 1998, 50, 1346-50.

18 GRATZ NG. Emerging and resurging vector-borne diseases. *Ann. Rev. Entomol.*, 1999, 44, 51–75.

19 GRISHIN E. Polypeptide neurotoxins from spider venoms. *Eur. J. Biochem.*, 1999, 264, 276-80.

20 GROSSMAN TH., KAWASSAKI ES., PUNREDDY SR., OSBURNE MS. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene*, 1998, 209, 95-103.

21 HERRMANN JL., DELAHAY R., GALLAGHER A., ROBERTSON B., YOUNG D. Analysis of post-translational modification of mycobacterial proteins using a cassette expression system. *FEBS Lett.*, 2000, 473, 358-62.

22 HINIKER A., BARDWELL JC. *In vivo* substrate specificity of periplasmic disulfide oxidoreductases. *J. Biol. Chem.*, 2004, 279, 12967-73.

23 JAKUBOWSKI JA., KELLEY WP., SWEEDLER JV. Screening for post-translational modifications in conotoxins using liquid chromatography/mass spectrometry: an important component of conotoxin discovery. *Toxicon*, 2006, 47, 688-99.

24 KADOKURA H., KATZEN F., BECKWITH J. Protein disulfide bond formation in prokaryotes. *Ann. Rev. Biochem.*, 2003, 72, 111-35.

25 KAGAN BL., BALDWIN RL., MUNOZ D., WISNIESKI BJ. Formation of ion-permeable channels by tumor necrosis factor-alpha. *Science*, 1992, 255, 1427–30.

26 KHAN SA., ZAFAR Y., BRIDDON RW., MALIK KA., MUKHTAR Z. Spider venom toxin protects plants from insect attack. *Transgenic Res.*, 2006, 15, 349-57.

27 LE COUTEUR DG., MCLEAN AJ., TAYLOR MC., WOODHAM BL., BOARD PG. Pesticides and Parkinson's disease. *Biomed. Pharmacother.*, 1999, 53, 122-30.

28 LIPKIN A., KOZLOV S., NOSYREVA E., BLAKE A., WINDASS JD., GRISHAN E. Novel insecticidal toxins from the venom of the spider *Segestria florentina*. *Toxicon*, 2002, 40, 125–30.

29 MANZOLI-PALMA MF., GOBBI N., PALMA MS. Insects as biological models to assay spider and scorpion venom toxicity. *J. Venom. Anim. Toxins*, 2003, 9, 174-85.

30 NAKAMOTO H., BARDWELL JCA. Catalysis of disulfide bond formation and isomerization in the *Escherichia coli* periplasm. *Biochim. Biophys. Acta*, 2004, 3, 111-9.

31 NARAYAN M., WELKER E., WEDEMEYER WJ., SCHERAGA HA. Oxidative folding of proteins. *Acc. Chem. Res.*, 2000, 33, 805-12.

32 NICHOLSON GM. Insect-selective spider toxins targeting voltage-gated sodium channels. *Toxicon*, 2007, 49, 490-512.

33 NORTON RS., PALLAGHY PK. The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon*, 1998, 36, 1573–83.

34 OERKE EC. Estimated crop losses due to pathogens, animal pests and weeds. In: OERKE EC., DEHNE HW., SCHÖNBECK F., WEBER A. Eds. *Crop production and crop protection-estimated losses in major food and cash crops*. Amsterdam: Elsevier, 1994: 72-741.

35 PENAFORTE CL., PRADO VF., PRADO MA., ROMANO-SILVA MA., GUIMARÃES PE., MARCO L., GOMEZ MV., KALAPOTHAKIS E. Molecular cloning of cDNAs encoding insecticidal neurotoxic peptides from the spider *Phoneutria nigriventer*. *Toxicon*, 2000, 38, 1443-9.

36 RASH LD., HODGSON WC. Pharmacology and biochemistry of spider venoms. *Toxicon*, 2002, 40, 225–54.

37 ROZHKOVA A., STIRNIMANN UC., FREI P., GRAUSCHOPF U., BRUNISHOLZ R., GRÜTTER G., CAPITANI G., GLOCKSHUBER R. Structural basis and kinetics of inter- and intra-molecular disulfide exchange in the redox catalyst DsbD. *Eur. Med. Biol. Org. J.*, 2004, 23, 1709-19.

38 SAMBROOK J., FRITSCH EF., MANIATIS T. Molecular cloning - A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 1989.

39 SOLLOD BL., WILSON D., ZHAXYBAYEVA O., GOGARTEN JP., DRINKWATER R., KING GF. Were arachnids the first to use combinatorial peptide libraries? *Peptides*, 2005, 26, 131–9.

40 SWIETNICKI S., PETERSEN R., GAMBETTI P., SUREWICZ WK. pH-dependent stability and conformation of the recombinant human prion protein PrP(90-231). *J. Biol. Chem*., 1997, 272, 27517–20.

41 UVERSKY VN., LI J., FINK AL. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. *FEBS Lett.*, 2001, 500, 105-8.

42 VONORAX EJ., TYLER MI., ATKINSON RK., HOWDEN MEH. Characterization of insecticidal peptides from venom Australian funnel-Web spiders. *J. Venom. Anim. Toxins incl. Trop. Dis.*, 2006, 12, 215-33.

43 WOJTASEK H., LEAL WS. Conformational change in the pheromone-binding protein from *Bombyx mori* induced by pH and by interaction with membranes. *J. Biol. Chem*., 1999, 274, 30950–6.