Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(6): 570-573
Received: 27-09-2019
Accepted: 30-10-2019

A Manivannan
Department of Agricultural Entomology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

KK Kumar
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

S Varanavasiappan
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

S Manimegalai
Department of Agricultural Entomology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

K Poornima
Department of Nematology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

C Devrajan
Department of Nematology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

D Sudhakar
Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

V Balasubramani
Department of Agricultural Entomology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

Corresponding Author:
V Balasubramani
Department of Agricultural Entomology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India

Expression, purification and bioassay of Cry55Aa protein against tomato root knot nematode, *Meloidogyne incognita*

A Manivannan, KK Kumar, S Varanavasiappan, S Manimegalai, K Poornima, C Devrajan, D Sudhakar and V Balasubramani

Abstract
A nematicidal Cry55Aa protein was expressed and purified from *E. coli* cells. Higher level of expression was observed in culture induced with 0.1mM of IPTG after four hours of induction. The Cry55Aa protein was purified by resuspending the crude protein in solubilizing buffer at pH 12 for 120 min. When *Meloidogyne incognita* was tested against different concentration of purified protein, a clear dose response lethality was observed with an LC_{50} value of 31.4 µg/ml.

Keywords: Cry55Aa protein, expression, purification, bioassay, *M. incognita*

Introduction
Bacillus thuringiensis (*Bt*), a gram-positive soil bacterium, produces crystalline inclusions during sporulation, having insecticidal proteins called δ-endotoxins. Different *Bt* strains produce a large number of insecticidal crystal proteins, which are encoded by *cry* genes and these strains are used as bio-pesticides for several decades [1]. Crystal proteins are known to be toxic against different insect orders such as Lepidoptera (butterflies and moths), Coleoptera (beetles) and Diptera [2], as well as nematodes [3]. Cry proteins such as Cry5, Cry6, Cry12, Cry13, Cry14, Cry21 and Cry55 are reported as nematicidal [4] and found to be toxic against several nematode genera including *Criconemella, Globodera, Ditylenchus, Heterodera, Meloidiogyne, Helicotylenchus, Pratylenchus, Rotylenchus, Radopholus, Tylenchus* and *Bursaphalenchus* [5]. *B. thuringiensis* strains produce more than one type of toxic proteins (cry, vip, cyt etc.) and to confirm the toxicity of a particular Cry protein, the respective *cry* gene is cloned and expressed in a heterologous bacteria like *E. coli* and used in bioassay studies [6].
E. coli is one of the most attractive hosts [7] for expression of heterologous protein with the advantages of fast growth in an inexpensive medium and the availability of a large number of cloning vectors [8]. In this study, an attempt was made to optimize heterologous protein expression in *E. coli*.
The plasmid pET29a containing *cry55Aa* gene under the control of a T7 promoter was used for heterologous expression of Cry55Aa protein. In order to increase the yield of the Cry55Aa protein, different concentrations of IPTG were used with different duration of induction. Expressed Cry55Aa protein was purified by using solubilizing buffer with different pH levels and incubation time. The purified Cry55Aa protein was tested against tomato root knot nematode, *M. incognita.*

Materials and Methods
Gene construct
The recombinant *E. coli* strain BL21 harbouring pET29a-*cry55Aa* gene (Accession no: HG764207.1) which was originally cloned from indigenous *Bacillus thuringiensis* isolate, T44 of *Bt* collections of CPMB & B, TNAU.

Expression of Cry55Aa in *E. coli* BL21 cell
Two *E. coli* cultures *viz*. BL21 (DE3) harbouring vector pET29a with *cry55Aa* insert and BL21 carrying pET29a vector without insert were grown in LB broth (5 ml) containing kanamycin 50 mg/l overnight at 37 ºC. About 500 µl of overnight-grown culture was inoculated in 25 ml of fresh LB broth with kanamycin 50 mg/l and incubated at 37°C in orbital shaker (180 rpm) till OD_{600} value reached ~0.6. Then different concentration of IPTG *viz*., 0.01, 0.05, 0.10, 0.50 and 1.0 mM was added to induce the protein expression and the cells were further grown at 37 °C for different durations (1, 2, 3, 4, 5 and 6 hrs) with a view to

optimize the IPTG concentration and duration of induction. The cultures were harvested by centrifugation and washed with 1X TE buffer **(**10.0 mM Tris-Cl, 1.0 mM EDTA, pH 8**)**. The pellet was dissolved in 5 ml of TE buffer containing 1 mM phenyl methyl sulfonyl fluoride and sonicated by using ultra-sonic liquid processor (Sonics and Material Inc., USA). Sonication was done with off pulsar mode for 4 min at 20 amplitude (4x 1 min with a time interval of 1 min.). The broken cells were pelleted by centrifugation at 7000 rpm for 15 min. The pellet was dissolved in the TE buffer and washed twice in the same buffer. The final product was suspended in 200 µl of sterile double distilled water containing 2mM PMSF and an aliquot of 5 µl was used for separation in SDS-PAGE.

Crystal protein purification

The crude Cry55Aa protein was collected by centrifugation and resuspended with solubilization buffer (8.7 mM tripotassium citrate, 43.4 mM citric acid and 10 mM dithiothreitol) at different pH levels (pH 9.0, 10.0, 11.0 and 12.0) and incubated at 37ºC for different durations (30, 60, 90 and 120 minutes) to solubilise the Cry55Aa protein. The contents were spun out at 8,000 rpm for 10 min and protein was precipitated from the supernatant by adding 1 M tripotassium citrate to adjust pH to 5.0 and incubated in ice overnight. The Cry55Aa protein was harvested by centrifugation for 10 min at 12,000 rpm. The precipitated Cry55Aa protein was washed with ddH_2O, dissolved in 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) (pH 8.0) and resolved in SDS-PAGE. The sds-page was performed as per the protocol given by Laemmli (1970) [9].

Lethal activity assay against *M. incognita*

Initial *M. incognita* culture was collected from infested tomato fields from Coimbatore, Tamil Nadu and multiplied in potted tomato plants maintained in greenhouse condition at 25 ± 2°C. The J2 stage of *M. incognita* were hatched on a filter paper which was saturated with water at 20-25 ^{0}C and used to test the toxicity of the Cry55Aa toxin and the assay was performed in 24 well plates. Each well with 400 µl S medium [10] contained purified Cry55Aa protein at 10, 20, 30, 40 and 50 µg/ml, about 100 J2 worms and 1 µl of chloramphenicol (10 mg/ml). BSA (20 µg/ml) was used as the control and each treatment was replicated four times. After incubation of 5 days at 25 ^{0}C, 50 µl culture from each well was transferred onto glass slides and observation made on live and dead worms under a dissecting microscope. Observation were made on at least three samples of 50 µl from each well.

Results and Discussion

Optimization of protein expression by IPTG induction

SDS-PAGE results showed that the protein expression increased gradually with increase in concentration of IPTG and maximum expression was observed with 0.1mM IPTG (Plate 1). The protein from recombinant *E. coli* BL21 (harbouring pET29a+*cry55Aa* gene) showed an expected prominent band of ~45 kDa on SDS-PAGE, which confirmed the *cry55Aa* gene expression, whereas no band was noticed in protein isolated from *E. coli* BL21 (harbouring pET29a vector alone). When IPTG (0.1 mM) induction was done for different durations (1, 2, 3, 4, 5 and 6 hrs) to check the level of expression, higher level of expression of protein was observed in cultures after four hours of induction (Plate 2).The present findings are comparable with previous report [11] suggesting that 0.1 mM IPTG was enough to induce the protein expression [12]. An IPTG concentration of 0.1mM and an induction period for four hours were found to be optimum for Cry55Aa expression in *E. coli* BL21 cells. Conditions for recombinant protein expression appears to be protein dependent [13]. For instance, induction with IPTG 10 µM was appropriate to induce the cHSPA6 expression which was 100 times less than generally used concentration and 5 h of post-induction incubation period was found to be better to produce folded cHSPA6 [14].

Purification of Cry55Aa protein by alkali solubilization

Solubility of crystal protein was found to have significant effect on toxicity [15]. Proteolytic activation of protoxin, is a crucial step in Cry protein toxicity mechanism, and found to be reliant on crystal protein dissolution in host intestine [16]. Some types of crystal protein from *B. thuringiensis* were found to be soluble at pH 12, making it nontoxic to insect with gut pH of 9.0 [17]. However, when crystal protein are presolubilized at high pH, these nontoxic crystal proteins exhibit significant toxicity to kill specific insect.

In this study, purification of Cry55Aa protein from recombinant *E. coli* (BL21) using solubilization buffer with different pH (9, 10, 11 and 12) and duration of induction (30, 60, 90 and 120 min) was attempted. Cry55Aa protein solubilization was maximum with pH 12, showing an expected prominent band of ~45 kDa on SDS-PAGE. When solubilization was done for different duration of incubation (30, 60, 90 and 120 minutes), solubilization was found to increase gradually from 30 to 120 minutes (Plate 3). The results are comparable with earlier reports on Cry5B (>pH 10), Cyt (pH 9.5), Cry4B (pH 12) [18, 19] and Cry6Aa2 (pH 9.5) [20].

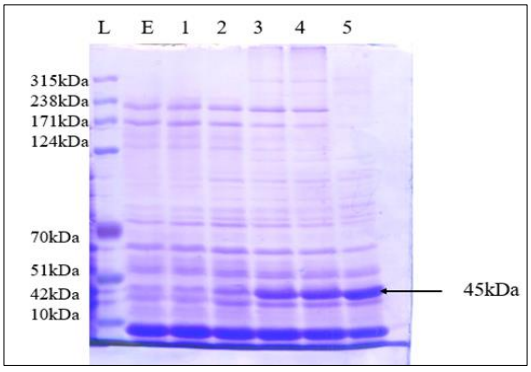

Plate 1: Standardization of IPTG concentration for Cry55Aa protein expression (L- Protein ladder (10-315kDa), E-empty vector (pET29a), 1 to 5- pET29a with Cry55Aa induced with IPTG 0.01, 0.05, 0.10, 0.50 and 1.0Mm)

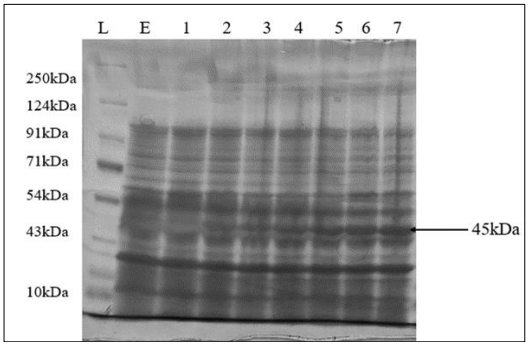

Plate 2: Standardization of induction period for Cry55Aa protein expression (L- Protein ladder (10-250kDa), E-empty vector (pET29a), 1 to 7- pET29a with Cry55Aa induced with 0.1mM IPTG for 0, 1, 2, 3, 4, 5 and 6 hrs)

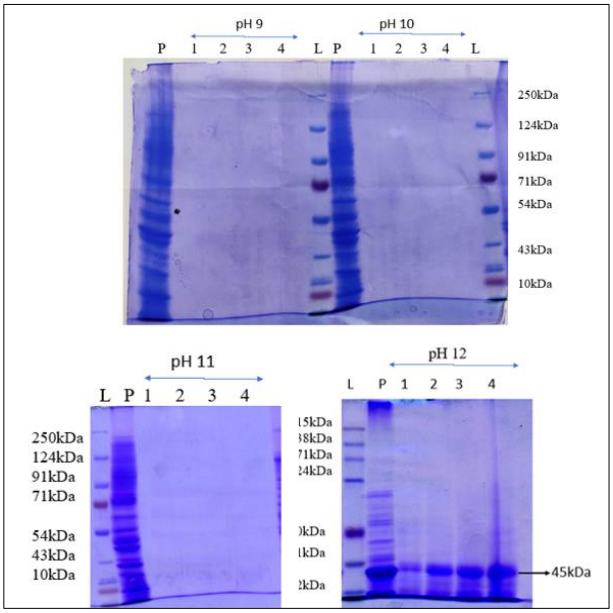

Plate 3: Purification of Cry55Aa protein from recombinant *E. coli* (L- Protein ladder, P- Protein without solubilization; 1 to 4- in each gel represent 30, 60, 90 and 120 minutes of incubation at 37 ºC)

Lethal activity of Cry55Aa protein on *M. incognita*

The per cent corrected mortality of Cry55Aa protein against *M. incognita* was 14.01, 30.61, 40.94, 48.51 and 58.81 at concentrations of 10, 20, 30, 40 and 50 µg/ml respectively (Table 1). Calculated LC_{50} value for Cry55Aa protein against second juvenile stage of *M. incognita* was 31.4 µg/ml (162.66–1109.62 µg/ml for 95% fiducial limits determined by profit analysis). The results are comparable with estimated LC_{50} values of Cry55Aa1 protein which was 23.2 µg/ml [21] against *M. hapla* as. In addition, the combination of Cry6Aa and Cry55Aa was reported to have toxicity on *M. incognita* [22]. The LC50 value of crude protein from *Bt* strain YBT-021 against *Tylenchorhynchus sp.*, *M. hapla*, *Pratylenchus scribneri*, *Ditylenchus destructor* and *Aphelenchoides sp.* was about 94.31 µg/ml, 35.62 µg/ml, 75.65 µg/ml, 215.21 µg/ml and 128.76 µg/ml respectively [23].

In the present study, have shown that the *cry55Aa* gene could be expressed in *E. coli* by inducing BL21-DE3 cells with 0.1 mM IPTG. The results presented here demonstrate that the Cry55Aa protein is toxic to *M. incognita*. This Cry55Aa is a potential nematicidal protein and could be included as a component of IPM, either as a formulated product or though transgenic means.

Table 1: Lethal activity assay of purified Cry55Aa protein against *M. incognita*

Treatment		Number of nematodes		Per cent mortality	Per cent corrected mortality	LC_{50}	Fiducial limit LC_{95}	
		Released	Dead				Lower	Upper
Cry55Aa protein (ug)	10	97.38	23.32	23.95 (29.29)^{c}	14.01	31.4	162.66	1109.62
	20	89.94	34.64	38.62 (38.40)^{b}	30.61			
	30	87.93	41.98	47.76 (43.71)^{b}	40.94			
	40	88.52	48.28	54.46 (51.46)^{a}	48.51			
	50	87.25	55.50	63.57 (52.98)^{a}	58.81			
Control		91.96	10.64	11.55 (19.80)^{d}				
CD (P=0.5)				7.20				

Mean of four replications. Values in parentheses are arcsine transformed. Means in a column followed by same superscripts are not significantly different at P≤0.05

Acknowledgement

The financial support received from the Department of Biotechnology, Government of India (Project No. BT/PR7742/PBD/16/1035/2013) is gratefully acknowledged.

References

1. Hofte H, Whiteley HR. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 1989; 53:242-255.
2. Sanahuja G, Banakar R, Twyman R, Capell M, Christou P. *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J. 2011; 9:283-300.
3. Wei JZ, Hale K, Carta L, Platzer E, Wong C, Fang SC *et al*. *Bacillus thuringiensis* crystal proteins that target nematodes. Proc. Natl. Acad. Sci. USA. 2003; 100:2760-2765.
4. Crickmore N. Using worms to better understand how *Bacillus thuringiensis* kills insects. Trends Microbiol. 2005; 13:347-350.
5. Edowaazn DE, Pein J, Sooresu JJ. Novel isolates of *Bacillus thuringiensis* having activity against nematodes. JP Patent, 1989, 1067192. http://www.wikipatents.com/JP-Patent1067192.
6. John CE. *Escherichia coli* as an Experimental Organism. In: eLS. John Wiley & Sons, Ltd: Chichester, 2014. DOI: 10.1002/9780470015902.a0002026.pub2
7. Swartz JR. Advances in *Escherichia coli* production of therapeutic proteins. Curr. Opin. Biotechnol. 2001; 12:195-201.
8. Jana S, Deb JK. Strategies for efficient production of heterologous proteins in *Escherichia coli*. Appl. Microbiol. Biotechnol. 2005; 67:289-298.
9. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680-685.
10. Larry J. Bischof, Danielle L. Huffman, Raffi V Aroian. Assays for Toxicity Studies in C. elegans With Bt Crystal Proteins. Methods and Applications: K, 2006, 1139-154. Totowa, Humana Press Inc.
11. Ariane LL, Júlia F, Gabriela SE, Daniel TV, Fernanda VR, Mitermayer GR *et al*. Evaluation of pre-induction temperature, cell growth at induction and IPTG concentration on the expression of a leptospiral protein in *E. coli* using shaking flasks and micro bioreactor. BMC Research Notes. 2014; 7(1):671.
12. Candan Y, Ilsen Z, Kirdar B. Optimization of starting time and period of induction and inducer concentration in the production of the restriction enzyme *Eco*RI from Recombinant *Escherichia coli* 294. Turk. J Chem. 1998; 22:221-226.
13. Sahdev S, Khattar SK, Saini KS. Production of active eukaryotic proteins through bacterial expression systems:

a review of the existing biotechnology strategies. Mol Cell Biochem. 2008; 307:249-264.
14. Malik A, Abdulrahman M, Alsenaidy Mohamed E, Wajahatullah K, Mohammed S, Alanazi, Mohammad DB. Optimization of expression and purification of HSPA6 protein from *Camelus dromedarius* in *E. coli*. Saudi J Biol. Sci. 2016; 23(3):410-419.
15. Aronson AI, Han ES, McGaughey W, Johnson D. The solubility of inclusion proteins from *Bacillus thuringiensisis* dependent upon protoxin composition and is a factor in toxicity to insects. Appl. Environ. Microbiol. 1991; 57:981-986.
16. Pardo LL, Muñoz GC, Porta H, Rodríguez AC, Soberón MB. Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*. Peptides. 2008; 30:589-595.
17. Du C, Martin PA, Nickerson KW. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. Appl. Environ. Microbiol. 1994; 60:3847-3853.
18. Samir N, Rumyana B, Rumyana K, Stefan D, Ivan M, Ruud AM. Solubilization, activation, and insecticidal activity of *Bacillus thuringiensis* Serovar thompsoni HD542 crystal proteins. Appl. Environ. Microbiol. 2008; 74(23):7145-7151.
19. Koller CN, Bauer LS, Hollingworth RM. Characterization of the pH-mediated solubility of *Bacillus thuringiensis* var. sandiego native delta-endotoxin crystals. Biochem Biophys Res. Commun. 1992; 30:184(2):692-9.
20. Hui L, Jing X, Qiaoni Z Liqiu X, Ziquan Y. The effects *of Bacillus thuringiensis* Cry6A on the survival, growth, reproduction, locomotion, and behavioral response of *Caenorhabditis elegans*. Appl. Microbiol. Biotechnol. 2013; 97:10135-10142.
21. Guo S, Liu M, Peng D, Ji S, Wang P, Yu Z, Sun M. New strategy for isolating novel nematicidal crystal protein genes from *Bacillus thuringiensis* strain YBT-1518. Appl. Environ. Microbiol. 2008; 74(22):6997-7001.
22. Peng D, Lujun C, Fenshan W, Fengjuan Z, Lifang R, Ming S. Synergistic activity between *Bacillus thuringiensis* Cry6Aa and Cry55Aa toxins against *Meloidogyne Incognita*. Microbial Biotechnology. 2011; 4(6):794-798.
23. Yu ZQ, Wang QL, Liu B, Zou X. *Bacillus thuringiensis* crystal protein toxicity against plant-parasitic nematodes. Chinese journal of Agricultural Biotechnology. 2008; 5(1):11-17.