African Journal of Biotechnology Vol. 10(65), pp. 14276-14285, 24 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1222
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Cloning and expression of the recombinant NP24I protein from tomato fruit and study of its antimicrobial activity

Mervat S. Mohamed[1], Walid Abu El-Soud[2*] and Magda F. Mohamed[1]

[1]Department of Chemistry, Faculty of Science, Biochemistry Speciality, Cairo University, Egypt.
[2]Department of Botany, Faculty of Science, Cairo University, Egypt.

Accepted 23 September, 2011

Thaumatin-like proteins (TLPs) constitutes a homogeneous family, members of which are produced by plants in response to different kinds of stress. NP24 protein is one of such salt-induced protein from tomato *(Solanum lycopersicum*) and it belongs to TLPs family. NP24 is a 24 kDa (207 amino acid) antimicrobial TLP found in tomato fruits. One isoform (NP24I) of NP24 was discovered in the outer pericarp of tomato fruit and is reported to play a possible role in ripening of the fruit in addition to its antimicrobial activity. In this study, the total RNA was isolated successfully from the outer pericarp of ripe (red) tomato fruit. cDNA was prepared and the gene coding for NP24I protein was amplified using conventional polymerase chain reaction (PCR). The gene was then cloned into Mach1™- T1® *Escherichia coli* cells, then subcloned into the over-expression vector pET-28a (+) using *BL21* expression bacteria. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the gene was over-expressed in *E. coli* as inclusion bodies. Optimization of the recombinant NP24I protein solubility was achieved by cold induction through decreasing both expression temperature and Isopropyl-beta-thio galactopyranoside (IPTG) concentration. The recombinant NP24I protein was purified using Ni-NTA resin, and then the antimicrobial activity of the purified recombinant NP24I protein was tested. The aims of this work were to study the cloning and expression of NP24 protein from local tomato cultivar in a prokaryotic system and to test the activity of the recombinant NP24, as well as to prove the activity of native protein on the bacterial as well as fungal growth.

Key words: Pathogenesis-related proteins (PR), low molecular weight thaumatin-like protein (TLPs), cloning, *Escherichia coli*, cold induction, antimicrobial activity, tomato *(Solanum lycopersicum).*

INTRODUCTION

The area of agricultural land in Egypt is confined to the Nile Valley and delta with a few oases and some arable land in Sinai. The total cultivated area is 7.2 million feddans (1 feddan = 0.42 ha), representing only 3% of the total land area. The entire crop area is irrigated except for some rain-fed areas on the Mediterranean coast. One of the important crops is the tomatoes, which is grown in three seasons; winter, summer and autumn, on about 3% of Egypt's total planted area. Losses in tomato crops have been large as a result of tomato leaf curl virus, early and late blight as well as nematodes.

The hypersensitive response of plants is one of the most rapid and efficient mechanisms for conferring a significant protection towards a broad range of phyto-pathogens which includes fungi, bacteria and viruses. In addition to the local responses which include local cell death at the site of infection (Van Loon and Van Strien, 1999) and metabolic changes of the cells surrounding the necrotic zone, the so-called pathogenesis-related (PR) proteins are expressed in the tissues. These PR proteins or at least some of them exhibited antimicrobial properties and it is generally believed that they contributed to disease resistance (Fritiget al., 1998). The pathogenesis-related protein families are classified into 14 groups and

*Corresponding author. E-mail: walidabc@gmail.com.

an additional three groups of PR proteins were proposed recently (Van Loon and Van Strien, 1999). The thaumatin-like proteins (PR-5) are classified as the fifth group of the PR protein family and so named because of the amino acid sequence homology to that of thaumatin, a sweet-tasting protein derived from *Thaumatococcus daniellii* (Eden et al., 1982).

NP24 is one of such salt-induced thaumatin-like protein that is found in suspension culture cells of tomato (*S. lycopersicum*) tissues (King et al., 1988) and it is a 24 kDa protein (207 amino acids). NP24 was first isolated, purified and crystallized from tomato fruits by Pressey (1997). A homologues protein was found to accumulate in cell suspension of tobacco (*Nicotiana tabacum*) under NaCl stress (King et al., 1986). Two isoforms (I and II) of the thaumatin-like protein NP24 was identified in tomato fruit (Pressey, 1997). NP24I protein is expressed mainly in the outer pericarp of healthy tomato fruits and its expression increases during ripening of tomato fruit until it reaches its maximum level in ripe (red) tomato fruit. However, NP24II expression increases slightly as the fruit begins to change colour and then remains constant (Pressey, 1997). Although the exact mechanism of action of the TLPs is yet unknown, it is believed that some TLPs are involved in plant defense (Van Damme et al., 2002). Some TLPs including NP24 was shown to possess glucanase activity (Grenier et al., 1999), which may be correlated with their antimicrobial properties. In the structure of tomato NP24I TLP, a prominent cleft is observed between domains I and II, a characteristic feature is observed in thaumatin as well as in the TLPs isolated from different plant species (Batalia et al., 1996; Ghosh and Chakrabarti, 2008; Koiwa et al., 1999; Min et al., 2004; Leone et al., 2006). The known TLPs are of molecular weights falling into two ranges; between 22 to 26 kDa and below 18 kDa. Most of the proteins in the high molecular weight group accumulated in cell vacuoles (Datta and Muthukrishnan, 1999). Proteins belonging to this group exhibited antimicrobial activities whereas TLPs of lower molecular weights are extracellular proteins and are found in cereals only, and there is no available evidence that these smaller TLPs have antimicrobial activities (Reimmann and Dudler, 1993).

This work stems from our interest to clone and express NP24 protein from local tomato cultivar in a prokaryotic system and to test the activity of the recombinant NP24 on the bacterial as well as fungal growth.

MATERIALS AND METHODS

All chemicals including kits, vectors and enzymes were purchased from Promega unless otherwise noted.

RNA isolation, cDNA synthesis and cloning

Total RNA was extracted from outer pericarp of the healthy ripe (red) tomato (*S. lycopersicum, ATLAS* seeds, BJ) fruit. Total RNA extraction was performed using Wizard ® Plus SV Total RNA isolation System. Fresh or frozen tissue (-80°C) was ground into a fine powder in liquid N_2 before the extraction buffer was added (1 ml of buffer to 1 g tissue). This method was rapid and it produced a large amount of high quality and undegraded RNA. To judge the integrity and overall quality of a total RNA preparation, the total RNA extracted was examined on denaturing formaldehyde/agarose gel (1.2% agrose in MOPS buffer) according to Lehrachet et al. (1977). First strand cDNA was synthesized using RT-PCR one step reaction Promega kit. cDNA region corresponding to matured tomato NP24I protein was amplified by PCR using specific primers, flanking the NP24 coding region and designed to create recognition sites for restriction enzymes *BamHI and HindIII* at 5' and 3' ends, respectively. The sequences of primers were: forward primer (M-F): 5' GGA TCC ATG GGC TAC TTG AC 3'; reverse primer (M-R): 5' AAG CTT TCA CTT GGC CAC TT 3'. The amplification reaction was carried out using the following PCR reaction conditions: (1) an initial denaturation step at 95°C (5 min); (2) 33 cycles of denaturation step at 95°C (1 min); (3) primer-specific annealing temperature at 45°C (1 min); (4) primer extension at 72°C (1 min) and (5) final extension at 72°C (7 min). PCR products were analyzed on 1% agarose gel in 1× Tris-borate EDTA (TBE) buffer (178 mM Tris base, 178 mM boric acid and 1 mM EDTA), extracted and purified from the agarose gel with Wizard® Plus SV DNA purification and clean up system. The purity and integrity of the NP24I PCR product was verified after extraction and purified by agarose gel electrophoresis. The purified DNA fragments were cloned into pGEM-T easy vector system, according to the manufacturer's instructions, then the (NP24I/pGEM-T) construct was introduced into *The Mach1™- T1® E. coli* chemically competent cells (Invitrogen, cat. No. ATCC#9637). Screening and selection of white/blue colonies was performed. The plasmids containing (NP24I/pGEM-T) construct were mini prepared using Wizard®Plus SV Mini Preps DNA Purification System, according to the manufacturer's instructions. Double digestion of the prepared plasmids by *BamHI* and *Hind III* was carried out at 37°C for 2 h in other to release the NP24 fragment and to verify that the plasmids contains NP24 insert.

DNA sequencing and analysis of the NP24 insert

DNA sequencing of NP24 insert was done to confirm the sequence of the cloned insert and to compare the sequence of NP24 from the local variety of tomato with the sequence of homologues NP24 from other varieties of tomato. Sequencing was done by dide-oxynucleotide chain-termination method and an automated sequencing system at Gene Analysis Unit, in VACSERA, Cairo, Egypt. The obtained sequences were analyzed for similarities to other known sequences found in the GenBank database using the BLAST program of the National Center for Biotechnology Infor-mation (NCBI) database.

Subcloning and expression of the recombinant tomato NP24I protein

The NP24I insert was subcloned into the T7-inducible vector, pET-28a (Novagen). Both the insert and pEt-28a vector were double digested with *BamHI* and *HindIII.* Then, each was purified and both were ligated using T4 DNA ligase (Promega), following the manufacturer's instructions. The ligation mix was then used to transform chemically competent *BL21 (DE3)-pLysS E. coli* (Invitrogen) according to Maniatis (1989). Successfully transformed *E. coli* cells containing the NP24/pET-28, a vector, were selected by plating the transformed cells on Luria broth (LB)/agar plates [1.0% (w/v) tryptone, 0.5 % (w/v) yeast-extract and 1.0% (w/v) NaCl at pH

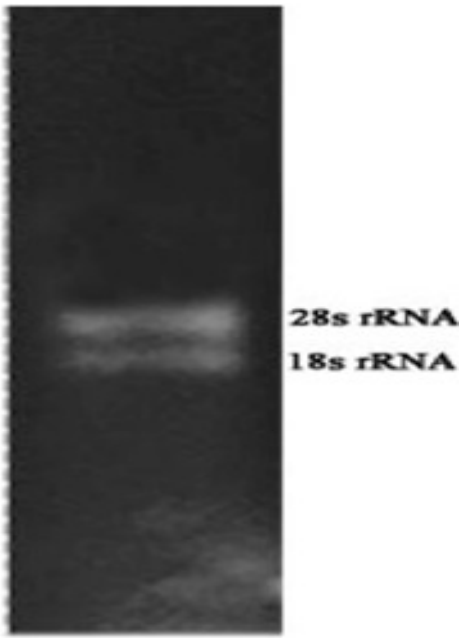

Figure 1. 1.2% denaturing formaldehyde agarose gel electrophoretic analysis of total RNA extracted from outer pericarp of mature tomato fruit.

7.5] and containing kanamycin (30 mg/ml). *E. coli* cells were induced to express NP24I protein by adding IPTG (1 mM final concentration) to the cultures when $O.D_{600}$ reached 0.6. The cells were induced for 4 h at 37°C, and then were collected by centrifugation at 6000 rpm for 10 min. The cells were resuspended in lysis buffer (10 mM Tris pH 7.5 and 100 mM NaCl) according to Reddy et al. (1996) and sonicated in ice bucket four times each for 30 s. The extract was centrifuged at 10000 rpm at 4°C for 10 min to separate the soluble proteins fraction (supernatant) from the insoluble proteins fraction (pellet). Both the soluble and insoluble protein fractions were analyzed for NP24I protein content by SDS-PAGE according to Laemmli (1970).

Optimization of recombinant NP24I solubility

Attempts and modifications were done during expression of the recombinant NP24I protein to direct the bacterial cells and to express NP24I protein as a native soluble protein. These attempts included changing the concentration of IPTG used in the induction process and/or changing the temperature at which the induction was carried out. The induction process was performed at 25°C overnight for about 16 h using different IPTG concentration (0, 0.05, 0.1, 0.3, 0.5 and 1 mM). The cells were collected by centrifugation at 5000 rpm for 10 min. Soluble proteins were extracted as mentioned earlier and analyzed on SDS- PAGE gel electrophoresis. After choosing the optimal induction conditions, scaling up of the expression conditions was done for the preparative expression of soluble NP24I protein.

Batch purification of the soluble 6× his-tagged recombinant NP24I protein from *E. coli* under native condition

The cell pellet that was prepared as mentioned earlier was resuspended in lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl and 10 mM immidazole, pH 8.0) at ratio of 2 to 5 ml per gram wet weight. Lysozyme was added to a final concentration of 1 mg/ml and incubated on ice for 30 min. The protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was then added to a final concentration of 3 mM to prevent protein denaturation. Total soluble proteins were extracted by sonication in ice bucket (4 times, 30 s each). The cell debris was removed by centrifugation at 5000 rpm for 15 min at 4°C; the supernatant containing the soluble NP24I was used directly or saved at 20°C until needed.

The purification of the recombinant NP24I protein from total soluble bacterial extract was performed using Ni-NTA agarose resin (QIAGEN) according to the manufacturer's instructions. The amount of cells required depends on the expression level of the 6× his-tagged protein and the expression system used. After batching the bacterial extract with Ni-NTA resin, the resin was washed with wash buffer (50 mM NaH_2Po_4; 300 mM NaCl and 20 mM immidazole, pH 8.0) and NP24I was then eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM immidazole, pH 8.0) in a minimal volume. The cells extract, the flow through and eluate were analyzed by SDS-PAGE for their content of NP24I protein.

Testing antimicrobial activity of the recombinant NP24I protein

The purified recombinant NP24I protein was tested for its bacterial and fungal growth inhibitory activity. Growth inhibition of the recombinant NP24I protein was determined as described by Roberts and Selitrennikoff (1990). 100 µl of an overnight culture of *Mach1™ - T1 ® E. coli* strain was spread on autoclaved LB/agar plate, and then leaved for 15 min to allow media absorb the bacterial culture. After this period, an autoclaved disk of filter paper saturated with a solution of the purified recombinant NP24I protein, or saturated with elution buffer to serve as a control was placed at the center of the plate. The plate was then incubated at 37°C overnight.

The antifungal effect of the recombinant NP24I protein was tested against fungal species *Fusarium oxysporum* and *Neurospora crassa* by placing a mycelial disc from an older culture of either of the earlier mentioned fungi on one side of the plate containing autoclaved DOX medium [sucrose (20 g/l), $NaNO_3$ (2 g/l), $MgSO_4$(0.5 g/l), KCl (0.5 g/l), K_2HPO_4 (1g/l) and agar (20 g/l)], while on the other side of the plate, a hole was dug with an autoclaved cork borer to place about 50 µl of the recombinant NP24I protein in it and left for 1 h to allow the media absorb the protein. The plate was incubated for five days at 28°C. A control experiment was carried out the same way, but using elution buffer in place of the purified NP24I to exclude any effect of the buffer components.

RESULTS AND DISCUSSION

Agarose gel analysis of the total RNA prepared from tomato fruit outer pericarp

The total RNA from the outer pericarp of ripe (red) tomato fruit (ATLAS seeds, BJ.) was extracted. The isolated RNA was analyzed on 1.2% denaturing formaldehyde/ agarose gel, to check the integrity of the preparation. Figure 1 shows two sharp bands on a background of faint smear. The upper sharp band corresponds to 28S rRNA species and it had almost twice intensity compared to the lower band which corresponds to 18S rRNA species. The background smear represented the rest of RNA population in the extract.

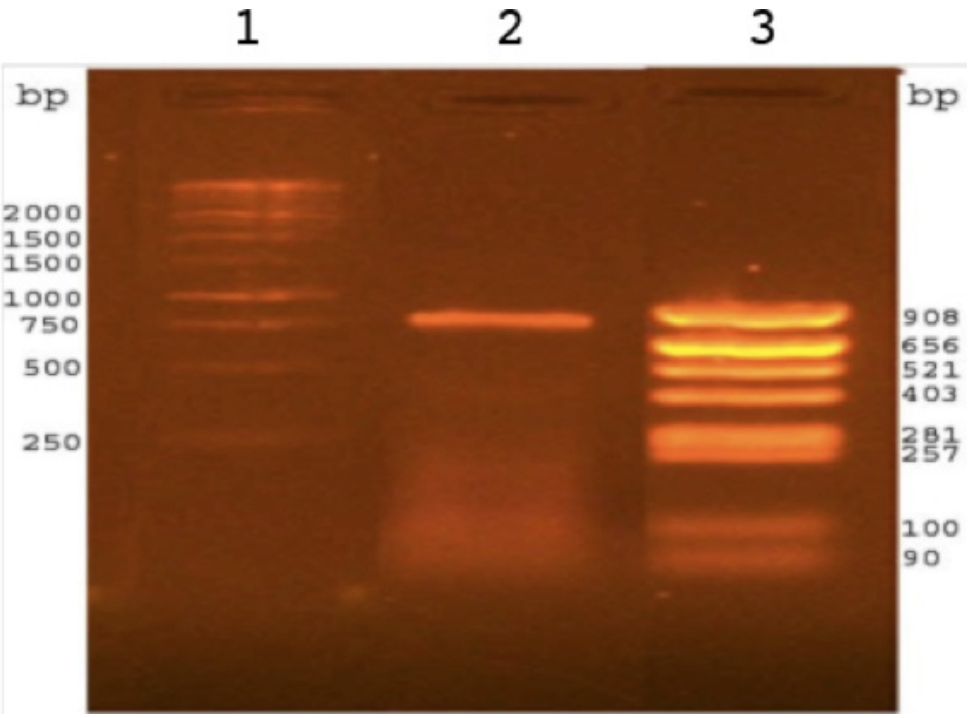

Figure 2. Agarose gel electrophoresis of PCR amplification of NP24 gene. The PCR product was analyzed by 1% agarose gel. Lane 1, 1 Kb DNA ladder molecular weight marker; lane 2, PCR product; lane 3, pBR322 DNA/AluI marker 20.

This result was as expected for RNA preparation from eukaryotic cells and this gave the confidence that the RNA preparation was intact and can be used for further experiments.

Amplification of the NP24I coding sequence

To amplify the NP24I coding sequence, the mRNA corresponding to the NP24I gene was reversely transcribed to corresponding cDNA. This was done using reverse transcriptase enzyme and specific primers flanking the NP24I coding region. After synthesizing the first strand cDNA, it was amplified using PCR. The PCR product was analyzed by agarose gel electrophoresis as shown in Figure 2. This result shows that a single specific PCR product was obtained with a molecular size of approximately 750 bp. This size was the expected size for NP24I coding region according to GenBank database on NCBI (M21346.1). The molecular size of the corresponding NP24I protein was in accordance with the estimated molecular sizes of other homologues TLPs, like that isolated from banana tissues (Barre et al., 2000).

Cloning and sequencing of NP24I coding region

The amplified NP24I coding sequence was ligated into pGEM-T cloning vector (Invitrogen) by TA ligation procedure, following the manufacturer's instructions. The ligation product was used to transform *The Mach1™- T1® E. coli* chemically competent cells. Successfully transformed cells were selected by blue/white selection, where white colonies were selected. Plasmid mini preparation were prepared and double digested with *BamHI/HindIII*. Agarose gel electrophoresis of digestion products was carried out. The gel (Figure 3) showed a lower band at approximately 750 bp corresponding to the NP24I insert and an upper band of a size more than 2000 bp corresponding to the linearized pGEM-T vector. These results indicate a successful ligation and cloning of the NP24I insert. Sequencing and blasting of the cloned NP24I (Figure 4) was inserted against non-redundant nucleotide database of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), which showed that the amplified sequence is the coding sequence of tomato NP24I gene.

After successful cloning of NP24I insert, it was double digested by *BamHI/HindIII* out of pGEM-T vector and ligated into the expression vector pET-28a (double digested with *BamHI/HindII)*. The ligation product was used to transform chemically competent *BL21 (DE3)-pLysS E. coli* cells. Successfully transformed cells were selected by growing the transformed cells on LB agar containing kanamycin. To confirm insertion of NP24I in pET-28a vector, plasmid mini preparations were prepared from transformed cells grown successfully in the presence of kanamycin. The plasmid mini preparation was double digested with *BamHI/HindIII* and digested

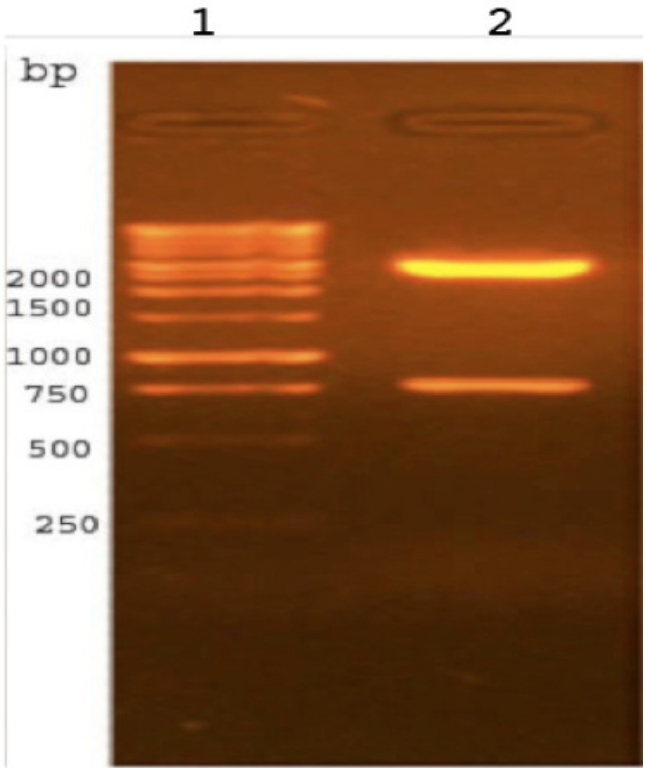

Figure 3. 1% agarose gel electrophoretic analysis of NP24I/pGEM-T construct double digested with *BamHI/HindIII.* Lane 1, 1kb DNA ladder marker; lane 2, NP24I/pGEM-T double digested construct.

1 ACTCTTGTCT CTTCTTCTTC TTTCCGACTT ATACANATGC TGCCACTATT GAGGTACGCA
ACAACTGTCC ATACACCGTT TGGGCGGCAT CGACTCCGAT 100

101 AGGCGGTGGT CGACGTCTCA ATCGAGGCCA AACATGGGTC ATCAATGCTC CGAGGGGAAC
TAAGATGGCA CGTATATGGG GTCGTACTGG TTGCAACTTT 200

201 AATGCTGCAG GCAGAGGTAC ATGTCAGACT GGTGATTGTG GTGGAGTCTT ACAGTGTACC
GGATGGGGCA AACCCCCAAA CACCCTAGCC GAGTACGCCT 300

301 TGGACCAGTT TAGCAACTTA GATTTTTGGG ACATTTCTTT AGTCGATGGA TTTAATATTC
CAATGACTTT TGCCCCAACA AAACCTAGTG GAGGAAAATG 400

401 TCACGCAATT CATTGCACGG CCAATATAAA TGGGTGGAAT GTCCTCGCCG CCCA 500

Figure 4. Partial sequence of NP24I coding sequence.

products were analyzed by agarose gel electrophoresis. Figure 5 shows two bands; a lower band of a size approximately 750 bp corresponding to NP24I insert and a upper one of around 5300 bp corresponding to the linearized vector. This result indicates a successful subcloning of NP24I insert into pET-28a vector.

Expression of recombinant NP24I protein

BL21 cells containing the NP24I/pET-28a construct were induced to express 6× his-tagged NP24I recombinant protein by adding 1 mM IPTG after the cells have reached linear phase of growth at 37°C. After 4 h of induction, the cells were collected by centrifugation and the cells pellet was resuspended in lysis buffer and extracted by ultrasonication. The insoluble protein and the cell debris were separated from the soluble proteins by centrifugation. The clear supernatant containing soluble proteins (soluble fraction) and pellet containing insoluble proteins (insoluble fraction) from induced and non-induced cells were analyzed by SDS-PAGE. Figure 6

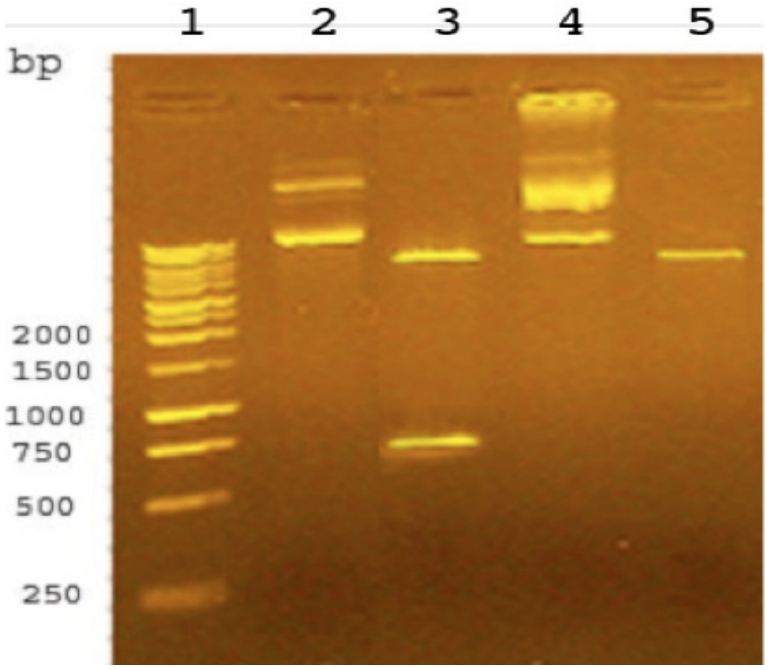

Figure 5. 1% agarose gel electrophoretic analysis of NP24I/pET-28a construct double digested with *BamHI/HindIII.* Lane 1, DNA molecular weight marker; lane 2: uncut NP24/pET-28a construct.; lane 3, digested construct (NP24/pET-28a) by *BamHI/Hind III*; lane 4, uncut pET-28a; lane 5, digested pET-28a by *BamHI/Hind III.*

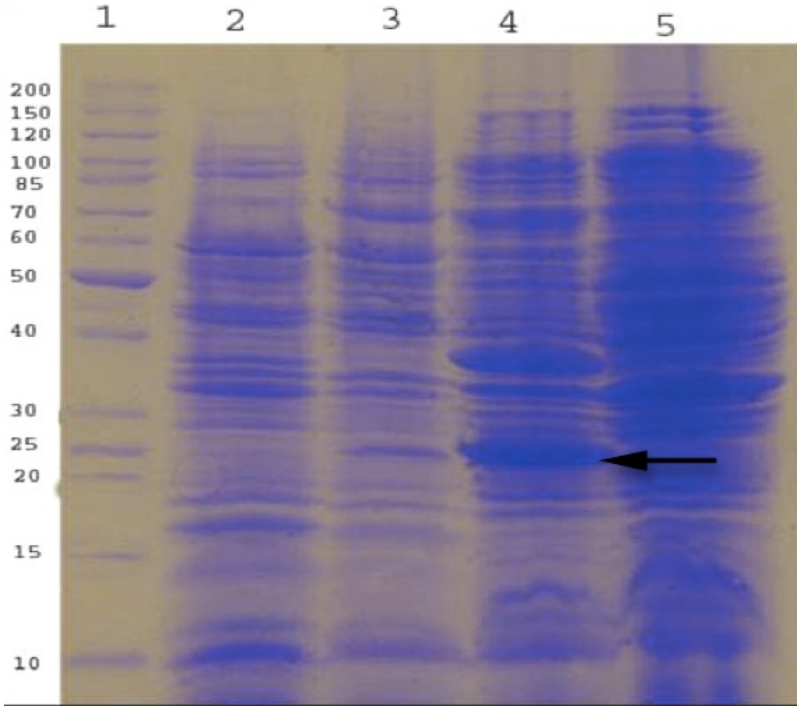

Figure 6. Coomassie blue stained SDS- PAGE of protein extract from transformed BL21 cells harboring NP24/pET-28a construct with and without induction with1 mM IPTG at 37°C. The arrow points to NP24I protein band. Lane1, protein marker molecular weight (from top 200 to 10 kDa); lane 3 and 2, soluble proteins extracted from BL21 cells with and without induction with 1mM IPTG respectively; lane 4 and 5, insoluble proteins extracted from BL21 cells with and without induction with 1mM IPTG, respectively.

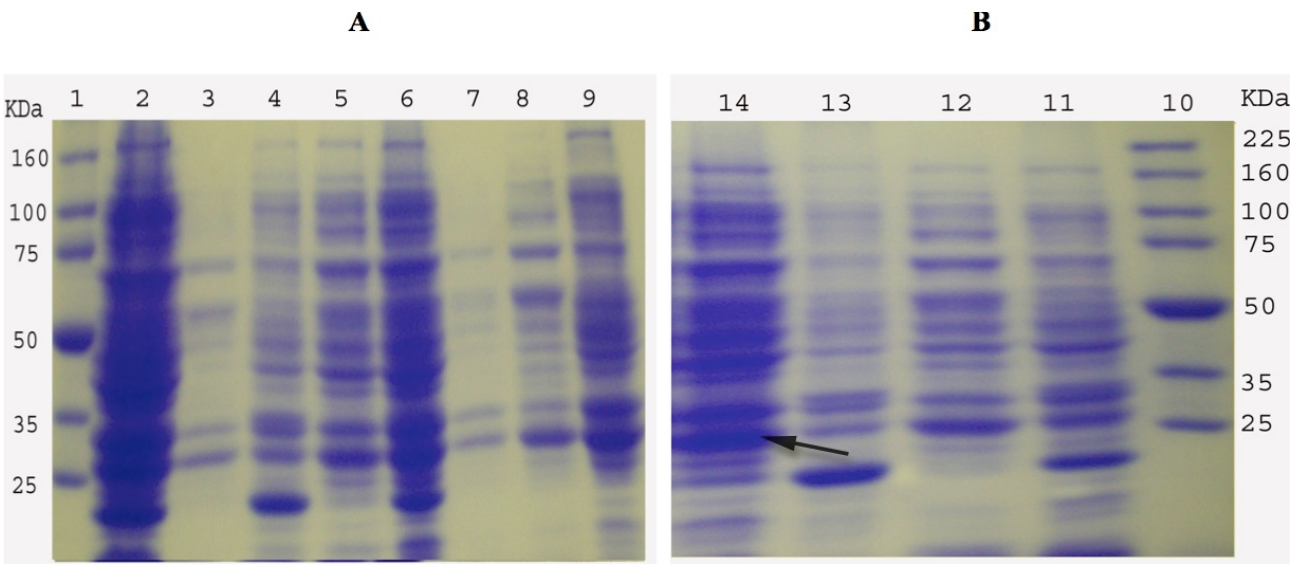

Figure 7. Coomassie blue stained SDS- PAGE of soluble and insoluble protein fractions of BL21 cells induced at 25°C using different IPTG concentrations. The arrow points to NP24I protein band. Lanes 1 and 10, protein size marker; lanes 2, 5, 6, 9, 11 and 13, insoluble protein fraction from BL21 cells induced with 1, 0.5, 0.3, 0.0, 0.1 and 0.05 mM IPTG, respectively; lanes 3,4, 7, 8, 12 and 14, soluble protein fraction from BL21 cells induced with 1, 0.5, 0.3, 0.0, 0.1 and 0.05 mM IPTG, respectively.

shows that a thick protein band of approximate molecular mass of 25 kDa was found in the insoluble fraction of induced cells but not in the non-induced cells fractions. A faint band of the same molecular weight was observed in the soluble fraction of induced cells. These results indicate that the recombinant NP24I protein expression in BL21 cells was not correctly folded and/or was toxic to bacteria cells. Hence, it aggregated and formed insoluble inclusion bodies. Similar results was recorded when the homologues thaumatin protein from *T. daniellii* was cloned and expressed in *Penicillium roquefortii,* where a recombinant protein of molecular size of 25 kDa was obtained in an insoluble form as inclusion bodies (Faus et al., 1997). In addition, when tomato and tobacco suspension cells were adapted for growth in the presence of NaCl or other water potential reducing agents, an abundant quantity of a 24 kDa protein was accumulated (King et al. 1986). The cDNA clones of these proteins were subsequently characterized (King et al., 1988; Singh et al., 1989) as NP24 and osmotin, respectively. In order to obtain recombinant NP24I in a soluble form, modifications of the expression and induction conditions were tried.

Optimization of the recombinant NP24I expression conditions

Attempts were made by modifying the induction conditions in order to obtain the recombinant NP24I in a soluble form. Lowering the IPTG concentration and/or modifying the induction temperature was carried out. After inducing the bacterial cells at 25°C and using different concentrations for IPTG (1, 0.5, 0.3, 0.1 and 0.05 mM), the soluble protein fraction was analyzed by SDS-PAGE. Figure 7a and b show that, by decreasing the temperature and lowering the IPTG concentration, more of the NP24I protein was expressed in the soluble form, and maximum amount of soluble form was obtained at 0.05 mM IPTG at 25°C. Lowering the induction condition temperature or IPTG concentration resulted in a lowered expression levels of the recombinant protein in *E. coli* cells. At such low level of expression, the bacteria can handle the expressed recombinant protein and fold it in a proper soluble form. Also, it was reported that several allergenic TLPs were present in fruits (Breiteneder, 2004) such as apple (Mal d 2), cherry (Pru av 2), bell pepper (Cap a 1 w), kiwi fruit (Act c 2), tomatoes (Lyc e NP24), etc.

Purification of recombinant NP24I protein from *E. coli* BL21 cells

A large scale (1 L) BL21 cells culture was induced under optimal conditions for getting recombinant NP24I in a soluble form. The recombinant NP24I protein was expressed from pET-28a vector as 6× his-tagged protein. After induction, the cells were extracted by ultrasonication and the recombinant NP24I was purified from the clear extract under native conditions using Ni-NTA IMAC (Immobilized Metal Affinity Chromatography), by patching technique according to the manufacturer's instructions (QIGEN). All of the purification steps were monitored using SDS-PAGE. Figure 8 shows that after purification, NP24I was obtained in a soluble form in a relatively high

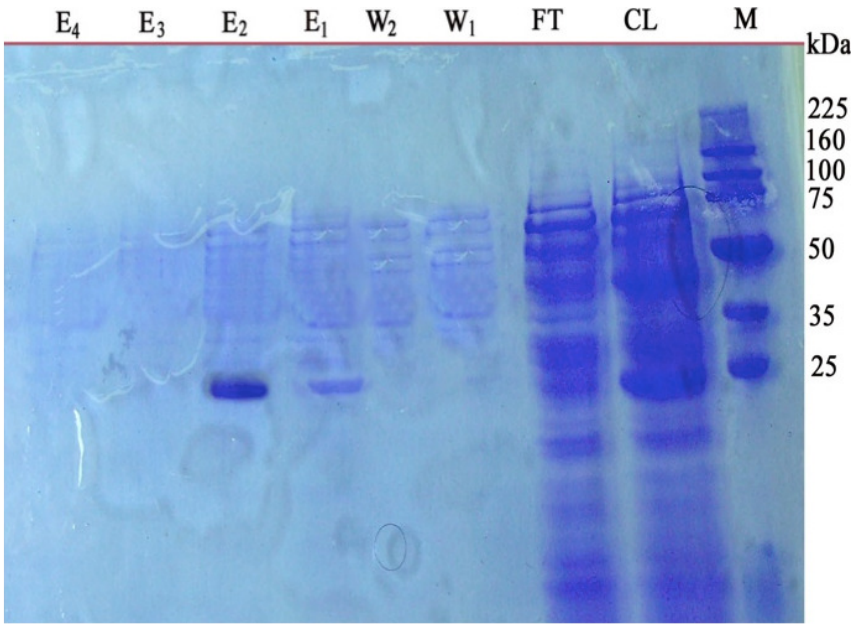

Figure 8. Coomassie blue stained SDS- PAGE of the purification steps of the recombinant NP24I protein under native condition. M, protein molecular weight marker; CL, total protein extract; FT, flow through; W_1, W_2, first and second washing steps, respectively; E1, E2, E3 and E4, first, second, third and fourth elution steps, respectively.

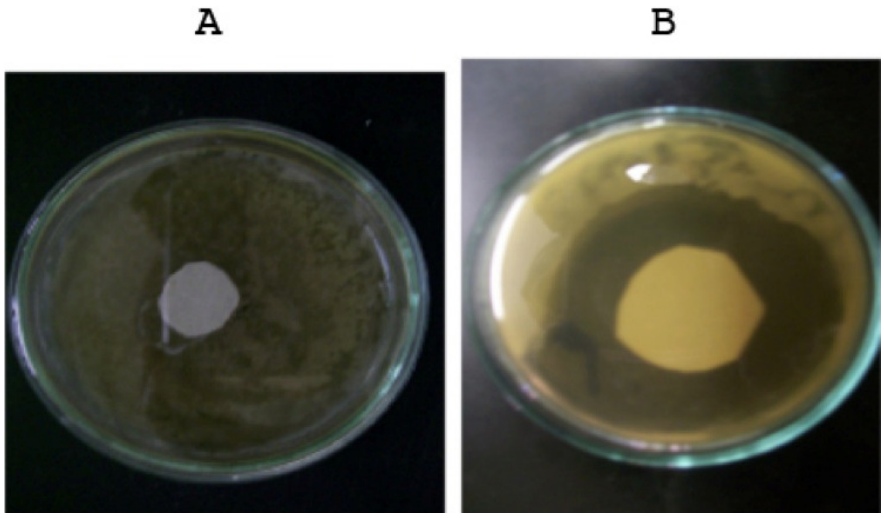

Figure 9. Test for the antibacterial activity of purified recombinant NP24I protein. (A) control plate; (B) test plate.

purity level. In addition, thaumatin-like proteins (PR-5) in tomato plants were detected in plastids where it specifically accumulated in chloroplast starch granules when plants were manipulated to express systemic acquired resistance (Jeun and Buchenauer, 2001)

The *in vitro* antimicrobial activity of purified NP24I

The purified recombinant NP24I protein was tested against some bacterial and fungal strains to prove its antimicrobial potency. When the recombinant NP24 protein was tested against The *Mach1™- T1® E. coli* strain, it was found that this *E. coli* strain is sensitive to NP24I protein as a clear inhibition zone appeared as shown in Figure 9b. A control Petri dish was prepared by placing a filter paper saturated with elution buffer instead of the recombinant NP24I protein as shown in Figure 9a, where the buffer did not affect growth of bacterial cells. This indicate that the recombinant tomato NP24I protein

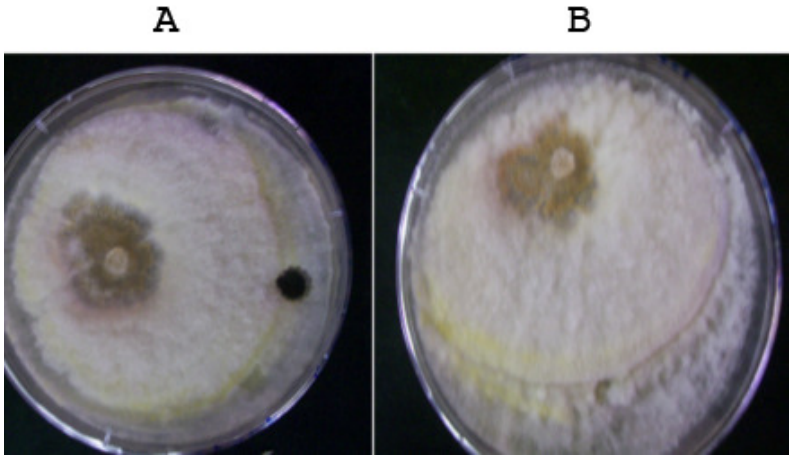

Figure 10. Test for the antifungal activity of purified recombinant NP24I protein. (A) Control plate; (B) test plate.

is a potent inhibitor for the growth of the *Mach1™- T1® E. coli* strain. However, when the recombinant NP24I protein was tested against the fungus *F. oxysporum*, it was found to be less potent toward it (Figure 10b). A control sample is shown in Figure 10a and this result is in accordance with the result obtained from banana thaumatin-like protein (Ban-TLP). When Ban-TLP was tested for antifungal activity *in vitro,* no antifungal effect could be observed towards *N. crassa* and *Fusarium culmorum* even when the protein was tested at a concentration of 250 µl/ml (Barre et al., 2000). Although the thaumatin-like fruit proteins from *Dyospyrose texana* (Wu and Huynh, 1994) and grapevine (Salzman et al., 1998) inhibited the growth of several phytopathogenic fungi, this antifungal activity cannot be extrapolated to all fruit-associated homologus because the TLP from cherry (Fils-Lycaon et al., 1996) and tomato NP24 TLP (this work) were devoid of antifungal activity, which made its general role as an antifungal agent unlikely.

Conclusion

The recombinant NP24I protein was successfully expressed in BL21 *E. coli* as a soluble protein and purified under native conditions. Its antibacterial activity was tested and it showed antibacterial activity against the experimental bacteria model *E. coli*. However, it showed no antifungal activity against *F. oxysporum.* The increase in NP24I expression level during ripening suggests a possible role in fruit development and ripening. Nevertheless, this study suggests a possible role of NP24 isolated from tomato pericarp in disease resistance.

REFERENCES

Barre A, Peumans WJ, Menu-Bouaouiche L, Van Damme EJM, May GD, Herrera AF, Leuven FV, Rougé P (2000). Purification and structural analysis of an abundant thaumatin-like protein from ripe banana fruit. Planta, 211: 791-799.

Batalia MA, Monzingo AF, Ernst S, Robertus JD (1996). The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 protein family. J. Nat. Struct. Biol. 3: 19-23.

Breiteneder H (2004). Thaumatin-like proteins a new family of pollen and fruit allergens. Allergy, 59: 479-481.

Datta SK, Muthukrishnan S (1999). Pathogenesis Related Proteins In Plants. Publisher CRC PRESS INC, ISBN No. 0849306973.

Eden L, Hesling L, Klock R, Ledeboer AM, Meat J, Toonen MY, Visser C, Verrips CY (1982). Cloning of cDNA encoding the sweet tasting plant protein thaumatin and its expression in *Escherichia coli*. Gene, 18: 1-12.

Faus I, Patino C, Del Rio JL, Del Moral C, Sisniega H, Rubio V (1997). Expression of a synthetic gene encoding the sweet tasting protein thaumatin *E.coli*. Biochemica Biophysical Res. Commun., 229: 121-127.

Fils-Lycaon BR, Wiersma PA, Eastwell KC, Sautiere P (1996). A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. Plant Physiol. 111: 268-273.

Fritig B, Heitz T, Legrand M (1998).Antimicrobial proteins in induced plant defense. Curr. Opin. Immunol. 10: 16-22.

Ghosh R, Chakrabarti C (2008).Crystal structure analysis of NP24-I: a thaumatin-like protein. Planta, 228(5): 883-890.

Grenier J, Potvin C, Trudel, J., and Asselin, A. (1999).Some thaumatin-like proteins hydrolyse polymeric b -1, 3-glucans. J. Plant, 19: 473-480.

Jeun YC, Buchenauer H (2001). Infection structures and localization of the pathogenesis-related protein AP24 in leaves of tomato plants exhibiting systemic acquired resistance against *Phytophthora infestans* after pretreatment with 3-aminobutyric acid or tobacco necrosis virus. J. Phytopathol . 149(3-4): 141-153.

King GJ, Hussey Jr. CE, Turner VA (1986). A protein induced by NaCl in suspension cultures of *Nicotianatabacum* accumulates in whole plant roots. Plant Mol. Biol. 7: 441-449.

King GJ, Turner VA, Hussey CE, Wurtele ES, Lee SM (1988). Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein. Plant Mol. Biol. 10(4): 401-412.

Koiwa H, Kato H, Nakatsu T, Oda J, Yamada Y, Sato F (1999). Crystal structure of tobacco PR-5d protein at 1.8 A ° resolution reveals a conserved acidic cleft structure in antifungal thaumatin- like proteins. J. Mol. Biol. 286: 1137-1145.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. J. Nat. 227: 680-5.

Lehrach HD, Diamond JM, Wozney Boedtker H (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. J. Biochem. 16: p. 4743.

Leone P, Menu-Bouaouiche L, Peumans WJ, Payan F, Barre A,

Roussel A, Van Damme E JM, Rouge P (2006). Resolution of the structure of the allergenic and antifungal banana fruit thaumatin like protein at 1.7-A°. J. Biochimie, 88: 45-52.
Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press. p. 1.74.
Min K, Ha SC, Hasegawa PM, Bressan RA, Yun DJ, Kim KK (2004). Crystal structure of osmotin, a plant antifungal protein. J. Proteins Struct. Funct. Bioinform. 54: 170-173.
Pressey R (1997).Two isoforms of NP24: a thaumatin-like protein in tomato fruit. J. Phytochem. 44: 1241-1245.
Reddy ASN, Safadi F, Narasimhulu S, Golovkin M, Hu X (1996). A novel plant calmodulin-binding protein with a kinesin heavy chain motor domain. J. Biol. Chem. 12: 7057-7060.
Reimmann C, Dudler R (1993). cDNA cloning and sequence analysis of a pathogen induced thaumatin-like protein from rice (*Oryza sativa*). Plant Physiol. 101: 1113-1114.
Roberts WK, Selitrennikoff CP (1990). Zeamatin, an antifungal protein from maize with membrane permeabilizing activity. J. Gen. Microbiol. 136: 1771-1778.
Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM, Bressan RA (1998). Coordinate accumulation of antifungal proteins and hexoses constitutes during fruit ripening in grape. Plant Physiol. 117: 465-472.
Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA (1989). Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol. 90: 1096-1101.
Van Damme EJM, Charels D, Menu-Bouaouiche L, Proost P, Barre A, Rouge P, Peumans WJ (2002). Biochemical, molecular and structural analysis of multiple thaumatin-like proteins from the elderberry tree (*Sambucusnigra L.*). J. Planta, 214: 853-862.
Van Loon LC, Van Strien EA (1999).The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. 55: 85-89.
Wu L, Huynh QK (1994). Isolation and characterization of a 27 kDa antifungal protein from the fruits of Diospyrostexana. Biochem. Biophy. Res. Commun. 202: 666-672.