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Induction of *Trichosanthes cucumerina* var. *anguina* Hairy Roots Using *Agrobacterium rhizogenes* ATCC 15834 for Production of Bioactive Protein

CHURIYAH[1*], GUSTAF ADOLF WATTIMENA[2], AND BAMBANG PONTJO PRIYOSOERYANTO[3]

[1]*Center for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology (BPPT), BPPT Building II, 15th floor, Jalan MH. Thamrin No.8, Jakarta 10340, Indonesia;*
[2]*Department of Agriculture, Institut Pertanian Bogor, Darmaga Campus, Bogor 16680, Indonesia;*
[3]*Department of Veterinery, Institut Pertanian Bogor, Darmaga Campus, Bogor 16680, Indonesia*

In the recent years, hairy roots have become a useful model system for the study of bioactive secondary molecule production and protein expression. Hairy roots of *Trichosanthes cucumerina* var. *anguina* were obtained by direct inoculation of the plantlet with *Agrobacterium rhizogenes* ATCC 18534 strain agropine. Six root clones of *Trichosanthes cucumerina* var. *anguina* hairy roots TH1, TH2, TH3, TH4, TH6, and TH8 were obtained from the induced hairy roots. The hairy root proteins were extracted with phosphate buffer saline, then fractionated by ammonium sulphate precipitation (80%), dialysis, and gel filtration. The toxicity of the proteins was analyzed using brine shrimp lethality test followed by cytotoxicity test (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay) using HeLa and K-562 cancer cell line. The Th2 root clone showed the highest protein yield (0.83%) and most toxic on BSLT with Lethal Concentration 50 was 5.76 µg mL^{-1}. PCR analysis indicated the integration of *rolB* gene to the genome of TH2 root clone which was showed by a DNA band of 780 bp on the electrophoretic agarose gel. Protein fractionation of the TH2 root clone resulted in four fractions, one of which the-TH2-3 protein fraction revealed the highest yield (0.29%) and toxicity on BSLT with LC_{50} up to 0.92 µg mL^{-1}. The Cytotoxicity test of the TH2 protein and TH2-3 protein fraction indicated that both of proteins inhibited the proliferation of Hela and K-562 cell with LC_{50} up to 49 µg mL^{-1}.

Key words: *Agrobacterium rhizogenes,* bioactive protein**,** hairy roots, *Trichosanthes cucumerina*.

Kultur akar rambut telah banyak digunakan sebagai model untuk mempelajari produksi berbagai senyawa metabolit sekunder serta ekspresi protein. Penelitian yang dilakukan bertujuan untuk mendapatkan kultur akar rambut dari *Trichosanthes cucumerina* var. *anguina* yang dapat memproduksi protein bioaktif melalui infeksi *Agrobacterium rhizogenes* strain ATCC 15834. Protein dari kultur akar rambut diekstraksi menggunakan dapar fosfat pH 7, dan difraksinasi dengan menggunakan pengendapan ammonium sulfat (80%), proses dialisis, dan kromatografi filtrasi gel. Protein dan fraksinya diuji toksisitasnya secara *brine shrimp lethality tes* (BSLT), kemudian protein dan fraksi protein yang aktif diuji sitotoksisitasnya secara *in vitro* menggunakan sel kanker HeLa (human cervic carcinoma) dan sel K-562 (human erythro leukemia) dengan 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide. Dari hasil penelitian diperoleh 6 klon kultur akar rambut yaitu TH1, TH2, TH3, TH4, TH6 dan TH8 yang tumbuh stabil. Diantara 6 klon tersebut, klon TH2 menghasilkan rendemen protein paling tinggi sekitar 0.83% dari total akar rambut yang diekstrak dan paling aktif dalam uji BSLT dengan LD_{50} 5.7 µg mL^{-1}. Hasil analisis secara PCR dari klon TH2 menunjukkan terdapatnya pita DNA 780 bp pada ekeltroforesis gel agarose, yang membuktikan terintegrasinya gen *rol B* pada genom akar rambut TH2. Dari fraksinasi protein klon TH-2 secara kromatografi gel diperoleh empat fraksi protein di mana TH23 yang paling tinggi rendemennya yaitu 0.29% dari total protein yang difraksinasi, dan paling aktif pada uji BSLT dengan LD_{50} 0.92 µg mL^{-1}. Uji sitotoksisitas secara *in vitro* dari protein klon TH2 dan fraksi protein TH2-3 menunjukkan kedua protein tersebut menghambat proliferasi sel Hela dan sel K-562 dengan IC_{50} paling tinggi, yaitu 49 µg mL^{-1}.

Kata kunci: *Agrobacterium rhizogenes*, kultur akar rambut, protein bioaktif, *Trichosanthes cucumerina*.

Agrobacterium rhizogenes is a common soil organism capable of entering the plant through a wound which results in the transfer of part of the Ri (root inducing) plasmid from bacterial cell to plant genome, causing the proliferation of adventitious root (hairy roots) at the site of infection. These hairy roots can be excised and cultivated indefinitely under sterile conditions (Ercan *et al*. 1999). The active compound from a plant can be directly extracted from intact plants or produced by *in vitro* culture. The advantages of the *in vitro* culture are that the culture is sterile and the environmental conditions are controllable. The hairy root culture, resulting from plant transformation using *Agrobacterium rhizogenes* have attracted considerable attention, because they are usually able to produce the same compounds that can be found in the normal roots of the parent plant (Park and Fucchini 2000). The patterns of alkaloid-like compounds obtained by thin layer chromatography in some hairy roots of several species of Mexican cacti were qualitatively similar between the transformed and non-transformed roots (Gonzales-diaz *et al*. 2006). Hairy roots have many potential advantages for bioactive protein production, including a low risk of contamination with potential animal pathogens, having

*Corresponding author, Phone/Fax: +62-21-3169505,
E-mail: churiyah_zafrollah@yahoo.com

the proper protein modification machinery and involving an inexpensive scale-up for commercial production (Ko *et al.* 2006). To elaborate hairy roots, as a distinctive production strategy for the commercial production of valued natural products, will also contribute to the environmental safety and resource-sparing technology for valued natural products for which native plant stocks may be limited (Bolivar 2007).

Trichosanthes cucumerina var. *anguina* is a tropical or subtropical plant. Its popular name is snake gourd. In Indonesia it is called Paria Ular. The plant is used as a vegetable and traditional medicine, the fruit is considered to be an anthelmintic, an emetic, and a purgative agent. The seed is said to have a cooling effect, and peptides in the plant are commonly used as an abortifacient (Gildemacher *et al.* 1994). Previous researchers found several bioactive proteins from this plant. TAP 29 (Trichosanthes anti-HIV protein, 29 kDa), which was isolated and purified from *Trichosanthes kirilowii*, is capable of inhibiting HIV-1 infection and replication (Huang *et al*. 1991). Karasurin, an abortifacient protein has been isolated from fresh root tubers of *T. kirilowii* Maximowicz var. japonicum Kitamura (Toyokawa *et al.* 1991). A type-I ribosome-inactivating protein (RIP) designated trichoanguin was purified from *Trichosanthes anguina* seeds, which strongly inhibited protein synthesis in a rabbit reticulocyte lysate (Chow *et al.* 1999). A genomic clone encoding trichobakin, a type I ribosome-inactivating protein has been isolated from the plant *Trichosanthes sp.* and was found to inhibit luciferase mRNA translation in the rabbit reticulocyte cell-free system (Chi *et al*. 2001). A putative ribosome inactivating protein (RIP) named T 33 was isolated from dried roots of *T. kirilowii* Maximovicz (Fei *et al*. 2004).

In order to elucidate other bioactive proteins, and to provide a system for large scale production of the target protein, hairy root induction using *A. rhizogenes* was investigated. The research was conducted to verify the possibility of obtaining hairy root lines of *T. cucumerina* using *A. rhizogenes*, that are capable of producing stable bioactive protein for medication.

MATERIALS AND METHODS

Agrobacterium Culture. *A. rhizogenes* strain ATCC 15834 was grown on YMA medium which consist of 10% (w/v) mannitol, 0.5% KH_2PO_4, 0.2% $MgSO_4 7H_2O$, 0.1% NaCl, 0.4% yeast extract, solidified with 12% bacto agar. This culture was incubated at room temperature for 3 days.

Establishment of Hairy Root Cultures. Seeds of *T. cucumerina* were obtained from the experimental field of Institut Pertanian Bogor. The seeds were surface sterilized in a 30% domestic bleaching agent (1% of sodium hypochlorite) for 2 minutes. Seeds were then washed three times with sterile distilled water followed by soaking for 1 min in 70% ethanol. Subsequently seeds were transferred to a 20% solution of domestic bleach (1% of sodium hypochlorite) containing 50 µL of Tween 20 and soaked for 15-20 min followed by three times washing with sterile distilled water. The seeds were then cultured on basal Murashige and Skoog (MS) medium containing 0.9% (w/v) agar. The seed culture was incubated for 30 days at 25 °C in a light/dark regime of 16/8 hours under a light intensity of 15 lux until the development of plantlets. Hairy root formation was induced by direct inoculation of *T. cucumerina* plantlet with *A. rhizogenes*. The adventitious roots arising on the wound site were excised and transferred to MS solid medium containing 250 mg L^{-1} cefotaxime for eliminating the *Agrobacterium*.

Extraction of Proteins. Protein was extracted according to the procedure developed by Di-Toppi *et al*. (1996), with some modification. Hairy roots were washed with tap water and ground in cold phosphate buffered saline, consisting of 0.14 mM NaCl in 5 mM pH 7 Na-phosphate buffer, homogenized in the same buffer in the ratio of 1:4 (w/v). Each homogenate was stirred overnight at 4 °C, and then filtered. Filtrate was then centrifuged at 10 000 g for 25 minutes. The protein was precipitated using 80% saturated ammonium sulfate, and the extracted protein was dialyzed against 5 mM pH 7 Na phosphate buffer using cellophane. After the insoluble material had been removed by centrifugation, the dialysates were then lyophilized.

PCR Analysis of Transformation. Plant genomic DNA for polymerase chain reaction (PCR) analyses was isolated according to Hamill and Lidgett (1991). Isolated DNA was analyzed by PCR for *rolB* gene, and DNA plasmid from *A. rhizogenes* strain ATCC 15834 was used as a positive control. The primers designed to amplify *rolB* were *rolB*1 (5-ATGGATCCC AAATTGCTATTCC-CCCACGA-3) and *rolB*2 (5-TA GGCTTCTTTCATTC-GGTTTACTGCAGC-3). PCR for *rolB* was carried out by amplification under the following conditions. Initial denaturation at 94 °C for 2 min and then denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 1 min for 30 cycles, with a final extension at 72 °C for 5 min. The amplicons were analyzed by electrophoresis on 2% agarose gel with 1000 bp molecular markers.

Gel Filtration Chromatography. Protein fractionation using gel filtration chromatography was done following the procedure of Di-Toppi *et al*. (1996). Crude protein was diluted on 5 mM pH 7 Na-phosphate

buffer, and subjected to gel filtration on Sephadex G-75, equilibrated and eluted in the same buffer. The excluded protein was detected on the basis of their absorbance at 280 nm wavelength, then the same fraction was pooled and lyophilized.

Brine Shrimp Lethality Test (BSLT). A batch of hatching brine shrimps was set up with the temperature between 28 and 30 °C, under conditions of 30-35 ppm salinity, pH range of 8-9, and strong aeration under continuous light condition. After these conditions were fulfilled, about one teaspoon of brine shrimp eggs was added into the system. After approximately 48 hours of hatching, the phototropic nauplii were collected with a pipette from the lighted side and concentrated in a small beaker and used for toxicity testing. Different concentrations (0, 10, 100, and 1000 µg mL^{-1}) of the protein samples were prepared. These protein samples were placed in separated test tubes, and twenty brine shrimps were transferred to each test tube using micro pipettes. After 24 hours the test tubes containing different concentrations of protein samples were observed, and the number of surviving naupliiin each test tube was counted. The % (percentage) lethality of nauplii was calculated with each concentration represents each sample. The value of Lethal Concentration (LC_{50}) was calculated using a Finny computer program.

Cytotoxicity Assay. HeLa (human servic carcinoma) cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 1% antibiotics (50 IU mL^{-1} penicillin and 50 µg mL^{-1} streptomycin) and 10% fetal bovine serum in a humidified incubator containing 5% CO_2 at 37 °C. Subcultures were obtained by trypsin treatment of confluent cultures. The K-562 (human erythro leukemia) cell line was grown in suspension in the same medium. The cells were plated in 100 µL of medium in 96 microwell plates at a density of 10^5 cells/well for HeLa and 5 x 10^4 cells/well for K-562, and the plates were placed in a 37 °C, 5% CO_2 incubator. Next day the cell culture medium was mixed with 100 µL/well medium containing the indicated concentrations (0,10, 20, 30,40 and 50 µg mL^{-1}) of protein in triplicate. After 72 hours of treatment, the cells were harvested. We compared the different concentration of each protein which inhibited the growth of both cell lines, using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] dye-uptake method. To each cell was added with 10 ul of 0.5mg mL^{-1} MTT and this maintained at 37 °C, in 5% CO_2 incubator for 4 hours to allow MTT to be converted into formazan crystals by reacting it with metabolically active cells. The reaction was stopped by adding acidic isopronanol (34 µL HCl in 10 mL isopropanol), and cell viability was measured at 570 nm using a plate reader (Wang *et al*. 2000). The cells without treatment were used as controls.

RESULTS

Establishment of Hairy Root Cultures. An infection of *T. cucumerina* explants with *A. rhizogenes* ATCC 15834 strain agropin resulted in the formation of hairy roots in 70% of the explants. In this research, root tips were individually inoculated in the MS medium containing 250 mg mL^{-1} of the antibiotic cefotaxime. After several subcultures, the bacteria-free clones obtained were transferred to the medium without antibiotics. The aseptic root clones which showed the most favorable growth characteristics were chosen for further investigation, those were six hairy root clones namely TH1, TH2, TH3, TH4, TH6, and TH8.

Extraction of Proteins. The result of protein extraction was described by protein yield, that is the ratio between the protein which resulted from the extraction process and the extracted material. The protein yields ranged from 0.5-0.8 % (Table 1), with the highest yield was 0.83% resulted from TH2 root clone, and the lowest one was 0.51% from TH6 root clone.

PCR Amplification. PCR performed using primer specific for the sequences in the *rolB* gene resulted in the amplification of a single amplicon with the expected size of 780 bp (Fig 1). The amplicons were detected on the lane 2 (DNA of *A. rhizogenes*) and lane3 (DNA of TH2 hairy root clone) but not detected on lane 4 (DNA of non-transformed root). This 780 bp amplicon obtained from PCR amplification confirmed the presence of the *rol B* gene which indicating integration of Ri T-DNA into the genomic DNA of TH2 root clone. Whereas non-transformed root (normal root) as a negative control did not show any positive signal for the *rolB* gene**.**

Gel Filtration Chromatography. The chromatography elution profile showed the fractionation of TH2 protein with separated into four peaks (Fig 2). All protein fractions was examined for their toxicities through BSLT, and the best fraction was selected according to the highest yield and toxicity

Table 1 Protein yields of the six hairy root clones of *T.cucumerina* var. *anguina* and their activities on brine shrimp lethality test (BSLT).

Root clone	Yield (%)	LC_{50} (µg mL^{-1})
TH1	0.70	7.54
TH2	0.83	5.76
TH3	0.65	16.48
TH4	0.56	14.55
TH6	0.51	10.11
TH8	0.74	5.63

Table 2 Protein yields of the protein fraction of TH2 root clone and their activities on brine shrimp lethality test (BSLT).

Protein fraction	Yield (%)	LC_{50} (µg mL^{-1})
TH2-1	0.056	5.60
TH2-2	0.061	5.36
TH2-3	0.290	0.92
TH2-4	0.061	4.99

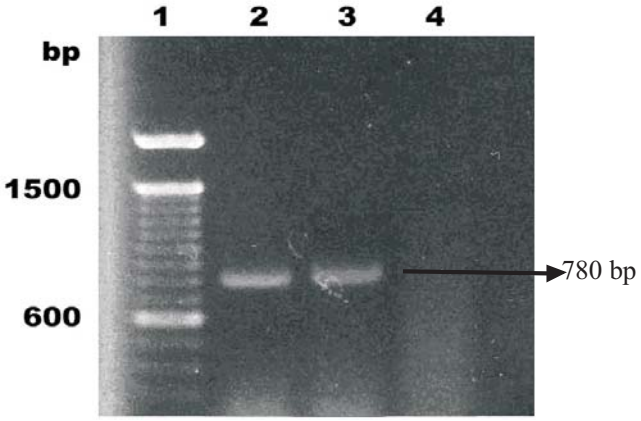

Fig 1 PCR amplification of genomic DNA from the TH2 root clones. (1) Molecular marker size, (2) Genomic DNA of *A. rhizogenes*, (3) DNA of TH2 hairy root clone, (4) DNA of the normal root.

on BSLT. The selected protein fraction was TH2-3 protein fraction which resulted in a protein yield of 0.29%, and its LC_{50} value was 0.92 µg mL^{-1} (Table 2).

Brine Shrimp Lethality Test (BST). The LC_{50} of the protein is defined as the concentration which kills, or inactivates by 50% of the tested brine shrimp. LC_{50} is inversely proportional to the toxicity of a compound, means that the lower is the LC_{50}, the higher is the toxicity. As listed in Table 1, each of the samples tested were toxic within 24 hours in different concentrations. The rate of lethality or mortality increased by increasing the concentration of the protein samples. The results indicated that before fractionation, protein

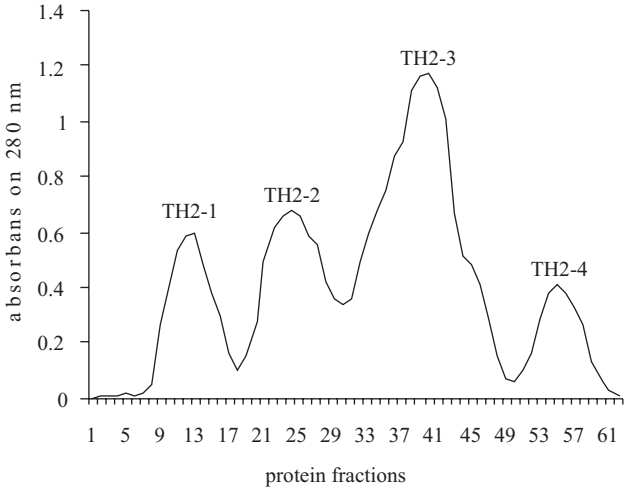

Fig 2 The chromatography elution profile of TH2 proteins on the chromatography gel filtration.

extracted from TH2 root clone was the most toxic protein against the brine shrimp nauplii compared with those extracted proteins from the other root clones. After fractionation the TH2-3 protein fraction was more active compared to the three other protein fractions. In the control test, all of the nauplii were alive after 24 hours. So it can be concluded that the toxicity that was found in the experiment might be due to the toxic property of the protein.

Cytotoxicity on Cancer Cell Line. The correlation between concentration of proteins and their cytotoxic effect on HeLa and K-562 cells was investigated by MTT assay. The protein at concentrations ranging from 1 to 5 µg mL^{-1} was effective in inhibiting the growth of the both cell lines tested for 72 hours. The TH2 protein inhibited proliferation of the HeLa cell in the range of 3.08 - 26.39 % and the K-562 cell in the range 5.68-31.66 % compared to the untreated cell. While the TH2-3 protein fraction showed more active in inhibiting of HeLa cell proliferation in the range of

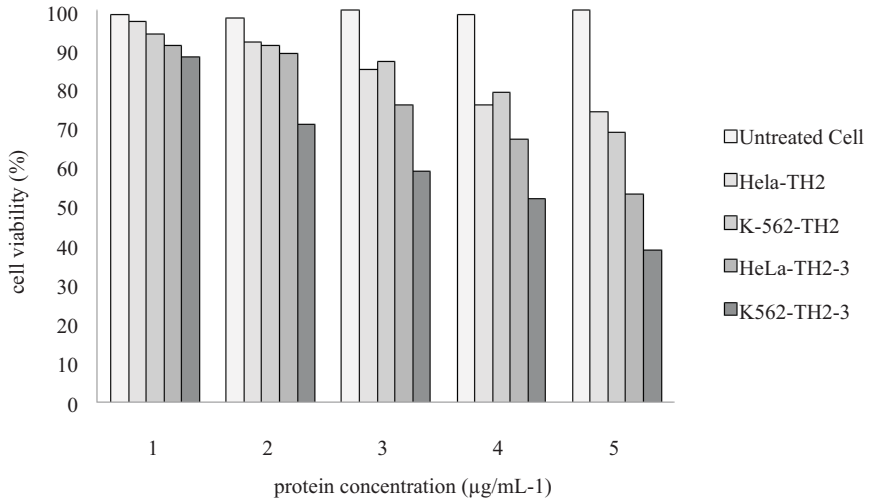

Fig 3 Comparison of the cytotoxicity of the TH2 protein and TH2-3 protein fraction on cancer cell line HeLa and K-562 *in vitro* using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method.

8.58 - 37.69 % and K-562 cell 12.28-61.15% compared to untreated cells. The inhibitory effect of the protein was dose-dependent (Fig 3). The protein additions gave different mortality rates at different concentrations, which increased with increasing concentration of protein.

DISCUSSION

We have observed that, among plants that produce bioactive compound, *Trichosanthes cucumerina* plant produced bioactive protein with highest yield and most active against cancer (Churiyah and Darusman 2009; Churiyah and Harran, 2010; Churiah and Sumaryono 2010). The research also showed that highest yield of proteins was from the seeds, then was from the root, and the lowest was from the fruit of *Trichosanthes cucumerina* var. *anguina* (Churiah and Sumaryono 2010). Therefore this plant was chosen as a model for inducing hairy root.

The hairy root cultures have excellences including stable in genetic, free of contaminants microbes, the environmental condition unrestrained, easily for extracting the proteins because have no chlorophyll as in the leaves of a plant, and not containing fat as on the seeds of plants.

A. rhizogenes, the causative agent of hairy root syndrome, is a Gram-negative soil bacterium, capable of entering plant through a wound and causing the proliferation of secondary roots. When the bacterium infects the plant, the T-DNA between the TR (T-DNA Right) and TL (T-DNA Left) regions of the Ri-plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable by-product, hairy roots, which will form at or near the site of infection (Ercan *et al*. 1999). *A. rhizogenes* infection resulting in the induction of roots that exhibited typical phenotypic characteristics of hairy roots such as intense hairiness, plagiotrophic growth, higher lateral branching and faster growth than the normal roots. Above all, the roots were able to grow well on hormone-free medium indicating the hormone-independent nature of roots that generally results is due to their transformed nature (Hamill and Lidgett 1997). The symptoms observed with *A. rhizogenes* are suggestive of auxin effects resulting from an increase in cellular auxin sensitivity rather than changed auxin production (Ercan *et al*. 1999).

A. rhizogenes ATCC 15834 strain agropin that was used in this research was found to be an effective means of inducing hairy-root formation. Previous studies have also revealed that the induction frequency of hairy root formation using this strain was higher than other *Agrobacterium* strains. Hairy root formation in some *Rubia tinctorum* populations have been induced by using many strains of Agrobacterium, in which the strain agropin 18534 showed the formation of hairy roots at the high frequency of up to 33-75% compared to the other strain R1000 which induced hairy roots at a lower frequency, about 15-50% (Ercan *et al*. 1999). The infection frequency of the *A. rhizogenes* strain 15834 on the opium popy (*Papaver somniforum*) resulted in hairy root formation up to 85% (Park and Fucchini 2000).

In our present study, of the six hairy- root clones established, there was a clear-cut difference from one clone to another in terms of protein production (protein yield) and the toxicity on BSLT (Table 1). Previous researchers evaluated the susceptibility of several species of Mexican cacti to *Agrobacterium rhizogenes*. Stem discs taken from *in vitro* cultured plants were inoculated with *Agrobacterium rhizogenes* A4 agropine-type strain. The frequency of hairy roots formation, the number of roots per explant and its growth rates were variable among the tested species (Gonzales-diaz *et al*. 2006). These differences may be attributed to secondary variation in the T-DNA insertion, the number of T-DNA copies that get integrated, and the size and location of integration of T-DNA of Ri -plasmid into the plant genome. Since the hairy root clone obtained by each transformed plant cell might result in a different clone, it is expected that each clone behaves differently with respect to growth and protein production. Therefore, the selection of a hairy root clones having properties of high biomass accumulation and protein production becomes one of the important and unexploited strategies to obtain a stable and high level of protein production. The best clone TH2 established in the present study produced protein about 0.85% from fresh weight extracted material, and its LC_{50} was 5.76 µg mL^{-1} on BSLT. Furthermore, after fractionation of proteins from TH-2 clones using gel-filtration chromatography the resultant TH2-3 protein fraction gave a high yield and was the most active in BSLT test than the other protein fractions. The yield was 0.29% from the weight of protein fractionated and its the LC_{50} was 0.92 µg mL^{-1}.

A. rhizogenes is known to lodge DNA plasmids having T-DNA region that gets transferred into the higher plant genome. The T-DNA has several genes, of which the important ones are the *rol* genes (*rol* A, B, C, D), *aux* genes involved in auxin metabolism and opine synthesizing genes. Since auxin is also synthesized by higher plants, the presence of the other genes such as *rol* genes and opine synthesis, the character of hairy roots are generally tested to confirm the integration of T-DNA into the host genome. In most

of the earlier studies, the transformation of plant tissues by *A. rhizogenes* was generally confirmed by using a biochemical method to detect opines in plant tissues. In the present study, the hairy root clones were tested for transformation and integration of T-DNA into the genome by means of PCR amplification using *rolB*-gene-specific-primer. Thus the presence of a *rolB* specific band upon PCR amplification clearly established the proof for successful transformation (Fig 1). The *rolA* gene was used to confirm the transformation of chicory hairy root genome (Park *et al*. 2002), and the *rolA*-gene also used to prove the successful transformation of hairy root cultures of red beet (*Beta vulgaris*) (Rudrappa *et al*. 2005). Another research group demonstrated the presence of the *rolB* genes in the DNA of the transformed roots (Gonzales-diaz *et al.* 2006).

To succeed in establishing a hairy root culture system for a certain plant species, several essential conditions should be taken into consideration. These conditions include the bacterial strain of *A. rhizogenes*, an appropriate explants, a proper antibiotic to eliminate bacteria, and a suitable culture medium (Bi Hu and Min Du 2006). Stable transformation and expression of transgene was achieved in Witloof chicory (*Cichorium intybus*) using an *A. rhizogenes* mediated system. Transformation frequency varies with the use of different types of strains of *A. rhizogenes*, concentration of *A. rhizogenes* and age of explants. Park *et al.* (2002) found a stable transformation and expression of transgene in Pokeweed (*Phytolaca americana*) hairy root and produce novel ribosome-inactivating protein in it culture.

The results of MTT assay indicated that both of the proteins were effectively inhibiting the growth of HeLa and K-562 cell, although the mechanism of action was not clear yet. Previous research reported that the active compound cucurbitacin isolated from *Trichosanthes kirilowii* acts as an inhibitory component on tyrosinase activity and melanin synthesis of B16/F10 melanoma cells (Hyuncheol *et al.* 2002) and induced apoptosis through caspase-3 and phosphorylation of JNK in HepG-2 hepatocellular carcinoma cells (Takashaki *et al*. 2009).

In conclusion, the present study demonstrates that *A.rhizogenes* 15834 strain Agropin used with *T. cucumerina* established hairy root cultures that can be useful for stable bioactive protein production.

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