Chapter 9

Production of Recombinant Cholera Toxin B Subunit in *Nicotiana benthamiana* Using GENEWARE® Tobacco Mosaic Virus Vector

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Abstract

Here, we describe a method to produce a recombinant cholera toxin B subunit in *Nicotiana benthamiana* plants (CTBp) using the GENEWARE® tobacco mosaic virus vector system. Infectious transcripts of the vector RNA are generated *in vitro* and inoculated on *N. benthamiana* seedlings. After 11 days, CTBp is extracted in a simple tris buffer at room temperature. No protease inhibitor is required. The leaf homogenate is treated with mild heat and a pH shift to selectively precipitate host-derived proteins. CTBp is purified to >95 % homogeneity by two-step chromatography using immobilized metal affinity and ceramic hydroxyapatite resins. This procedure yields on average 400 mg of low-endotoxin CTBp from 1 kg of fresh leaf material.

Key words Plant-made pharmaceutical, *Nicotiana benthamiana*, Plant virus vector, Ceramic hydroxyapatite chromatography, Cholera toxin B subunit

1 Introduction

Cholera toxin B subunit (CTB) is a 55-kDa homopentameric, non-toxic, GM1-ganglioside-binding subunit of the holotoxin. Due to potent mucosal immunogenicity, the protein induces robust systemic and mucosal antibody responses upon oral administration. These antibodies are capable of neutralizing cholera holotoxin [1], the virulence factor responsible for the severe diarrhea symptom of cholera. Hence, a recombinant CTB produced by bacterial fermentation is used as a component of the internationally licensed oral cholera vaccine Dukoral®. In addition to mucosal immunostimulatory effects, a number of studies have shown that CTB elicits anti-inflammatory and regulatory T-cell responses, suggesting that the protein may be utilized for the development of immunotherapeutics against allergies and autoimmune diseases (reviewed in: [2, 3]).

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Plant-based expression systems provide an alternative biomanufacturing platform for recombinant proteins, with potential advantages in cost-effectiveness and scalability [4]. We have developed an expression and purification scheme to produce a recombinant CTB containing an Asn4 → Ser mutation and a C-terminal Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) extension in *Nicotiana benthamiana* plants (CTBp) utilizing the GENEWARE® tobacco mosaic virus (TMV) vector expression system. The Asn4 → Ser mutation eliminates eukaryote-specific *N*-glycosylation at Asn4 upon expression in plant cells, while the KDEL endoplasmic reticulum retention signal increases recombinant protein accumulation. These amino acid sequence modifications do not affect CTB's GM1-ganglioside binding affinity, physicochemical stability, or oral immunogenicity [5].

The GENEWARE®expression system takes advantage of the functions of fully infectious TMV, including cell-to-cell and systemic movement activities that are mediated by movement protein (MP) and coat protein (CP), respectively. GENEWARE®also exploits the strength and duration of the viral subgenomic promoter, and its ability to reprogram the translational priorities of the plant host cells so that virus-encoded proteins are synthesized at high levels, similar to TMV CP [6, 7].

We have developed a simple extraction and two-step purification scheme to successfully produce high-purity CTBp from fresh leaf material of *N. benthamiana* plants within 11 days of inoculation. After extraction, a series of mechanical processes with mild heat treatment (55 °C) and pH adjustment are employed to remove host-derived impurities and thereby simplify downstream purification. Immobilized metal affinity chromatography (IMAC) and ceramic hydroxyapatite (CHT) chromatography are used to further purify CTBp. CTB is known to bind to immobilized Ni^{2+} ions through internal histidine resides [8]; therefore CTBp can be purified to high purity using IMAC. CHT is a multimodal resin that utilizes cation exchange and metal affinity, and is known to offer unique selectivity and often separates biomolecules that appear homogenous using other chromatographic methods.

The expression and purification scheme described herein allows for an easy and efficient way to manufacture CTBp with low endotoxin contamination, which may facilitate the research and development of mucosal vaccines and immunotherapeutics.

2 Materials

Prepare all solutions using ultrapure MilliQ water (MilliQ Synthesis, Millipore, 18.2 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 RNA Transcription

1. GENEWARE®vector DNA plasmid (Kentucky Bioprocessing, Inc.) containing the coding sequence for CTBp with rice α-amylase signal peptide (obtained via standard molecular biology/subcloning procedures using *Pac* I/*Avr* II restriction sites based on pNM156 [5]).
2. mMessage mMachine® Kit (Ambion).
3. 0.7 % standard agarose gel, and materials for gel electrophoresis.

2.2 N. benthamiana Inoculation

1. *N. benthamiana* plants seeded in 4 in. pots.
2. Diurnal growth chamber with constant humidity (23 °C, >50 % humidity, 16 h day/8 h night).
3. Frog Eye Soup (FES): 112 mM glycine, 60 mM potassium phosphate dibasic, 22 mM sodium pyrophosphate decahydrate, 1 % bentonite, 1 % celite. Add approximately 250 mL water to a 1 L graduated cylinder or glass beaker. Weigh 2.52 g glycine, 3.136 g potassium phosphate dibasic, 3.0 g sodium pyrophosphate decahydrate and transfer to the cylinder. Stir to dissolve. Add water to a final volume of 300 mL then add 3 g bentonite and 3 g celite. Store FES at 4 °C (*see* **Note 1**).

2.3 CTBp Extraction

1. Extraction buffer: 20 mM Tris–HCl, 500 mM sodium chloride, 20 mM ascorbic acid, 10 mM sodium metabisulfite, pH 5.0. Weigh 2.42 g tris(hydroxymethyl)aminomethane, 29.22 g of sodium chloride, 3.52 g of ascorbic acid, 1.9 g of sodium metabisulfite and transfer to a cylinder containing 900 mL of water (*see* **Note 2**). Stir to dissolve. Adjust the pH to 5.0 (*see* **Note 3**) using 1 M NaOH. Bring the volume to 1 L with water.
2. Industrial blender.
3. Cheese cloth.
4. Miracloth.
5. 250 mL Centrifuge Bottles.
6. Water bath set at 55 °C.
7. 10 N NaOH.
8. Centrifuge.
9. 0.22 μM Bottle top filter unit.

2.4 Purification

1. IMAC Buffer A: 20 mM Tris–HCl, 500 mM sodium chloride, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl) aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Bring the volume to 400 mL with water to make 50 mM tris buffer.

Adjust the pH to 8.0 using 1 M NaOH. Weigh 29.22 g of sodium chloride and transfer it to the cylinder containing 400 mL of 50 mM tris buffer. Add water to a volume of 900 mL. Stir to dissolve. Mix and adjust pH if needed using 1 M HCl. Add water to a volume of 1 L.

2. IMAC Buffer B: 20 mM Tris–HCl, 500 mM sodium chloride, 150 mM imidazole, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl)aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Add water to 400 mL to make 50 mM tris buffer. Adjust the pH to 8.0 using 1 M NaOH. Weigh 29.22 g of sodium chloride and 10.2 g of imidazole and transfer them to the cylinder containing 400 mL of 50 mM tris buffer. Add water to a volume of 900 mL. Stir to dissolve. Mix and adjust pH using 1 M HCl if needed. Add water to 1 L.
3. CHT Buffer A: 10 mM Tris–HCl, 5 mM sodium phosphate, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl) aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Add water to 400 mL to make 50 mM tris buffer. Adjust the pH to 8.0 using 1 M NaOH. A stock solution of 0.1 M sodium phosphate monobasic is made using 13.8 g/L and a stock solution of 0.1 M sodium phosphate dibasic (heptahydrate) is made using 26.8 g/L. Mix 5.3 mL of sodium phosphate monobasic and 94.7 mL of sodium phosphate dibasic. Add water to 200 mL to make 50 mM phosphate buffer. Adjust the pH to 8.0 using 1 M HCl. In a cylinder, mix 200 mL of 50 mM Tris–HCl with 100 mL of 50 mM phosphate buffer. Add water to a volume of 900 mL. Stir to dissolve. Mix and adjust pH if needed using 1 M NaOH. Add water to 1 L.
4. CHT Buffer B: 10 mM Tris–HCl, 250 mM sodium phosphate, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl) aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Add water to 400 mL to make 50 mM tris buffer. Adjust the pH to 8.0 using 1 M NaOH. A stock solution of 1 M sodium phosphate monobasic is made using 138 g/L and a stock solution of 1 M sodium phosphate dibasic (heptahydrate) is made using 268 g/L. Mix 13.25 mL of sodium phosphate monobasic and 236.75 mL of sodium phosphate dibasic. Add water to a volume

of 500 mL to make 0.5 M phosphate buffer. Adjust pH to 8.0 using 1 M HCl. In a cylinder, mix 200 mL of 50 mM Tris–HCl with 500 mL of 0.5 M phosphate buffer. Add water to a volume of 900 mL. Mix and adjust pH if needed using 1 M NaOH. Add water to 1 L.

5. ATKApurifier 100 liquid chromatography system (General Electric Company), or similar.
6. Talon® Superflow Metal Affinity Resin (Clontech).
7. CHT™ Ceramic Hydroxyapatite, Type I, 40 μM (Bio-Rad).
8. XK 26/20 column (GE Healthcare Life Sciences).
9. XK 16/20 column (GE Healthcare Life Sciences).
10. 15 % Tris-Glycine gels (Lonza) and materials for SDS-PAGE.

2.5 Formulation

1. Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium.
2. Amicon Ultra centrifugal filter, 30 K (Millipore).
3. 15 % Tris-Glycine gels (Lonza) and materials for reducing and non-reducing SDS-PAGE.

3 Methods

3.1 Capped Transcription Reaction Assembly

1. Synthesize 7-methyl guanosine capped RNA in vitro using the mMessage mMachine kit, according to the manufacturer's directions. For capped transcription reaction assembly, use 1 μg GENEWARE® vector DNA plasmid (not linearized), without [α-32P]UTP tracer, and incubate the reaction for 2 h at 37 °C. Add 1 μL of TURBO DNase, mix well and incubate 15 min at 37 °C.
2. Run a standard 0.7 % agarose DNA gel to check the transcript. A band should be visible at approximately 10,000 bp.

3.2 Inoculation of N. benthamiana Plants with GENEWARE® Transcripts

1. For each plant to be infected, create 100 μL of inoculum containing 0.1 μL of transcript (obtained from Subheading 3.1) and 99.9 μL of FES (*see* **Note 1**).
2. Inoculate two leaves (*see* **Note 4**) per plant by pipetting 25 μL of inoculum at two different locations per leaf (Fig. 1a).
3. While wearing gloves, hand rub the inoculum into the leaves (Fig. 1b).
4. Keep plants in a growth chamber at 23 °C and >50 % humidity, with a 16 h daytime/8 h nighttime schedule, for 11 days. Water plants immediately after infection and then every other day with tap water.

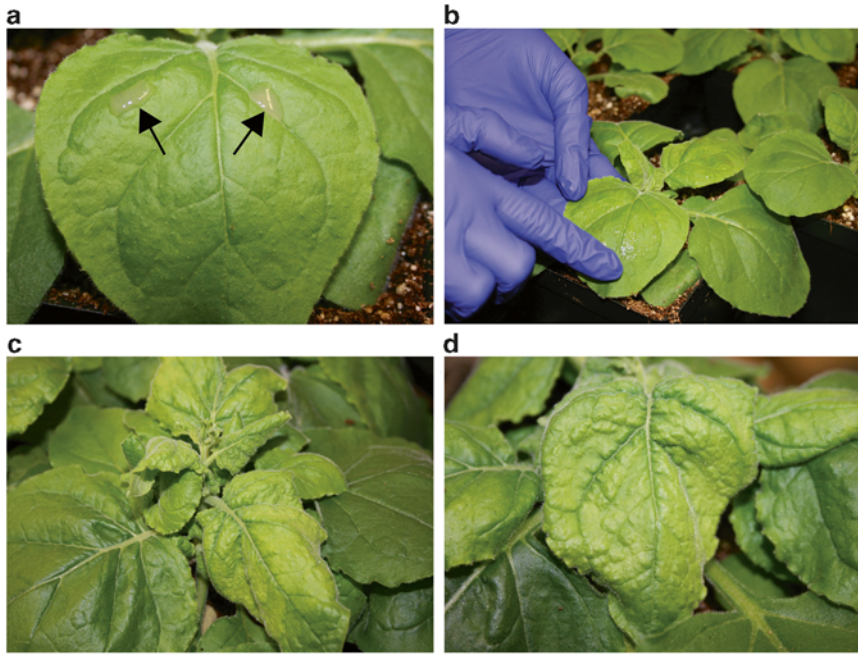

Fig. 1 Vector inoculation and infection of *N. benthamiana* plants with GENEWARE® transcripts. (**a**) Inoculation of the third and fourth leave down from the apical meristem. A hundred microliters of inoculum contains 0.1 μL of transcript and 99.9 μL of FES. An aliquot of 25 μL is pipetted (*black arrows*) in two different locations per leaf. (**b**) The inoculum is hand rubbed into the leaves. (**c** and **d**) TMV infection is manifested by the mosaic, crinkled phenotype

3.3 Extraction of CTBp from N. benthamiana Plants

The extraction procedure is performed at room temperature.

1. At 11 days post inoculation, harvest the infected leaves (*see* **Note 5**; Fig. 1c, d) expressing CTBp by cutting the leaves at the base of the stem and measure the total mass.
2. Immediately (*see* **Note 6**) add 2 mL of extraction buffer for every 1 g of leaf tissue, and mix in an industrial blender until the mixture is homogenous.
3. To remove the majority of the plant tissue, filter the extract through two layers of cheese cloth on top of one layer of miracloth. Collect the supernatant ("green juice") in sterile 250 mL centrifuge bottles.
4. In the centrifuge bottles, heat the extract for 25 min at 55 °C in a water bath (*see* **Note 7**) to allow host-derived proteins and starches to fall out of solution.
5. Centrifuge at 15,000 × *g* for 15 min at 4 °C.
6. Pour supernatant into a clean beaker. While mixing, pH the extract up to pH 8.0 by using 10 N sodium hydroxide. This pH shift facilitates the precipitation of host-derived impurities.

7. Centrifuge at 15,000 × *g* for 15 min at 4 °C.
8. Pass the supernatant through a 0.2 μM bottle top filter using a house vacuum.

3.4 Purification of CTBp from Clarified Extract

The purification procedures are performed at room temperature and CTBp purified product is stored at 4 °C until use.

3.4.1 IMAC

1. Pack the Talon Superflow Metal Affinity Resin in an XK 26/20 column to a 10 mL bed volume on an AKTA purifier.
2. Equilibrate the column with 10 column volumes (CV) of IMAC buffer A at 10 mL/min.
3. Load the clarified extract at a flow rate of 5.0 mL/min followed by a 10 CV wash with IMAC buffer A at 10 mL/min.
4. Elute proteins using a single-step gradient to 100 % IMAC buffer B for 5 CV at 10 mL/min. Collect 10 mL fractions.
5. Analyze each fraction for CTBp purity by SDS-PAGE using 15 % Tris-Glycine gels.
6. Combine pure CTBp-containing fractions for CHT purification (*see* **Note 8** and Fig. 2a).

3.4.2 CHT

1. Pack the CHT Hydroxyapatite, Type I, 40 μm resin in an XK 16/20 column to a 10 mL bed volume on an AKTA purifier.
2. Equilibrate the column with 10 CV of CHT buffer A at 10 mL/min.
3. Load the CTBp IMAC elution at a flow rate of 5.0 mL/min followed by a 10 CV wash with CHT buffer A at 10 mL/min.
4. Elute proteins using a gradient from 0 to 100 % CHT buffer B over 20 CV at 10 mL/min. Collect 10 mL fractions.
5. Analyze each fraction for CTBp purity by SDS-PAGE using 15 % Tris-Glycine gels (Fig. 2a).
6. Combine pure CTBp-containing fractions.

3.5 Formulation of CTBp

1. Ultrafiltrate and diafiltrate the CTBp (combined fractions from CHT purification in Subheading 3.4.2) into sterile Dulbecco's PBS (DPBS) using Amicon Ultra-15 30,000 MWCO centrifugal devices according to the manufacturer's instructions.
2. To determine the concentration of CTBp solution, measure the absorbance at 280 nm by UV-Vis spectroscopy. Use DPBS as a blank. Divide the absorbance value by CTBp's theoretical extinction coefficient at 280 nm of 0.7660 $(mg/mL)^{-1} cm^{-1}$.
3. Analyze the purity and pentamer formation of purified CTBp by use of an overloaded (5 μg) Coomassie-stained sodium

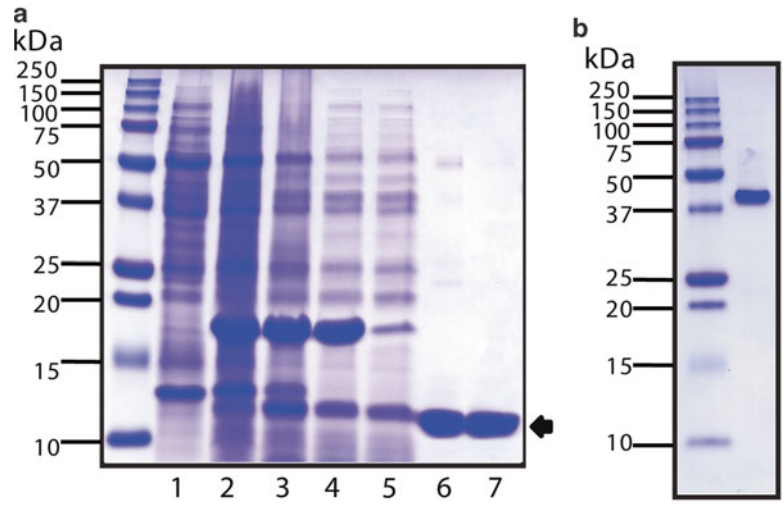

Fig. 2 Expression, clarification, and purification of CTBp. (**a**) Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude extracts and purified CTBp. Lane 1: Non-infiltrated leaf extract (extracted the same way as CTBp in Subheading 3.3 to create non-infiltrated green juice). Lane 2: CTBp expressing leaf extract ("green juice"). Lane 3: CTBp extract after heating at 55 °C for 25 min. Lane 4: CTBp extract after pH adjustment up to pH 8.0. Lane 5: CTBp after centrifugation and filtration (IMAC load). Lane 6: Five micrograms of CTBp purified by IMAC only. Lane 7: Five micrograms of CTBp purified by IMAC plus CHT. CTBp monomer is shown at 12.3 kDa, indicated by a *black arrow*, and was purified to >95 % purity after IMAC only as well as IMAC plus CHT. A major band at ~18 kDa in Lanes 2–4 corresponds to TMV CP. (**b**) Non-heat non-reducing SDS-PAGE analysis of 5 μg of CTBp purified by IMAC plus CHT. After both purification steps, CTBp was purified to >95 % purity while retaining pentamer formation (the protein migrates faster than the theoretical molecular size of approximately 60 kDa under the non-denaturing conditions)

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-heat non-reducing conditions using 15 % Tris-Glycine gels (*see* **Note 9**; Fig. 2a, b).

4 Notes

1. FES has settlement during storage. Mix well immediately prior to use to make the solution homogeneous.
2. Buffers with ascorbic acid and sodium metabisulfite are made immediately prior to use to prevent oxidation of the ascorbic acid and sodium metabisulfite.
3. The pH of the extraction buffer is prepared outside the typical buffering range of Tris–HCl. This is to incorporate the pH transition step prior to IMAC purification (*see* Subheading 3.3,

step 6), which allows for the precipitation of starches and thereby aids in the downstream column purification process. We have found that during the short period of extraction the pH does not change drastically. The "green juice" ends up with a pH of approximately 5.3.

4. The two leaves chosen for inoculation are the third and fourth leaves down from the apical meristem.
5. Inoculated leaves are distinguishable by the mosaic, crinkled phenotype.
6. Once extracted, we have found that it is best to clarify and purify immediately. Leaving the extract at 4 °C overnight or freezing at −20 °C results in a significant loss of CTBp.
7. Start timing the 25 min when the extract reaches 55 °C. Generally, it takes approximately 20 min for the extract to reach 55 °C.
8. CTBp is purified to >95 % purity after IMAC purification (*see* Fig. 2a). The additional CHT step is performed to remove residual small molecules from the plant extract.
9. We routinely obtain approximately 400 mg of purified CTBp from 1 kg of fresh leaf material using the procedure described here. In addition to SDS-PAGE analysis, GM1-ganglioside-binding affinity can be confirmed using GM1-ganglioside capture enzyme-linked immunosorbent assay or surface plasmon resonance [5].

Acknowledgments

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