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Process for production and purification of Lethal Toxin Neutralizing Factor (LTNF) from *E. coli* and its economic analysis

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Abstract

BACKGROUND—Purification of peptides offers unique challenges with respect to obtaining the desired process yield and selectivity. Lethal Toxin Neutralizing Factor (LTNF) is a peptide that is known to neutralize snake venom in mice when the peptide is preincubated with the venom prior to intravenous injection. A process for producing highly purified recombinant LTNF has been developed. The process has been modelled in SuperPro designer using laboratory data for a plant capable of producing 10 Kg of purified rLTNF. Economic analysis has been performed for manufacturing 3 ton of purified rLTNF

RESULTS—The process developed produces peptide in the form of concatemer that has been specifically designed to accumulate as insoluble inclusion bodies (IB) during expression in *E. coli*. A cation exchange chromatography step has been developed to capture the rLTNF concatemer at 140 g/L dynamic binding capacity. Further, the purified concatemer is cleaved completely into monomeric rLTNF using alpha-chymotrypsin enzyme. Finally, a reversed phase high performance liquid chromatography has been designed to purify rLTNF with a recovery of more than 90% and purity greater than 98%. The overall process recovery is 78±2% resulting in 3.36 g of purified product per batch. Techno-economic evaluation of the process has been performed to demonstrate its economic feasibility against currently marketed antivenom products.

CONCLUSIONS—The developed process is able to produce purified rLTNF with 78±2% recovery. The study shows that recombinant technology can be used to produce rLTNF cost effectively and shows potential as a substitute for currently available antivenoms against snakebite.

Keywords

Lethal Toxin neutralizing factor (LTNF); Concatemer; Peptide; Reverse phase high performance liquid chromatography (RP HPLC)

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INTRODUCTION

Snakebite has been a neglected disease for decades until the World Health Organization (WHO) included it in the list of tropical neglected diseases in 2009.¹ Snakebite can cause tissue necrosis, severe hemorrhaging, paralysis, heart failure, and other significant effects depending on the type of snake and severity of bite.^{2–5} The estimated number of envenomings around the world is as high as 1.84 million, resulting in death of more than 1 million people every year.^{6–9} There are several reports suggesting the need for a global initiative to monitor, control, and treat snakebite.^{10,11} Existing treatments for snake bites include heat treated multivalent antisera produced from horse or sheep.^{5,12} Current manufacturing practices for producing these antisera involve envenoming of horses with a specific venom pool to generate antibodies that are further isolated, purified, and lyophilized to obtain the antivenom in a powder form.^{12,13} While these antivenoms are effective against the different types of venoms (cytotoxic, hemotoxic, neurotoxic), there are inherent limitations and disadvantages associated with these treatments. The venom pool used for immunization depends on the geographical region, habitat, seasonal variations, and venom content of particular medically important species in the pool. Since the pool impacts specificity of the antivenom, it is the vital raw material for production and this creates challenges for manufacturing.¹³ Moreover, a large number of patients suffer from side effects including serum sickness, inflammation, acute reactions, vomiting, and fever.^{13–15} In spite of efforts to develop new antivenoms and to improve existing treatments, traditional methods of production, distribution, and treatments are being followed today in various parts of the world. There is a dire need for designing a process for manufacturing antivenom that follows the principles of good manufacturing practice (GMP) and can be used for production of safe and efficacious antidote for combating envenomation.

A peptide segment of a 68 kDa protein, named Lethal Toxin Neutralizing Factor (LTNF) has been identified from the opossum (*Didelphis virginiana*) serum.^{16–19} This peptide has been shown to exhibit activity as metalloproteinase inhibitor.¹⁷ Interestingly, the first 15 amino acids of the N-terminal sequence of the full protein were found to contain the activity of the complete protein. Since the opossums are known to possess a natural protection from snake bites, LTNF was examined and shown to be capable of neutralizing the toxic effects of venom from various snakes, insects, plant and bacterial derived toxins.²⁰ From a digest of the LTNF protein, researchers have reported that the N-terminal portion (10–15 amino acids) of the protein maintained the potency of the full protein when administered to mice.^{17,18}

A prior publication from our group has demonstrated the activity of LTNF against rattlesnake venom.²¹ The objective of current work is to develop a feasible process for manufacturing of highly purified rLTNF. Another objective of the paper is to perform a techno-economic evaluation of the process to examine its economic feasibility against currently available antivenoms.

MATERIALS AND METHODS

Materials

Glucose, kanamycin, isopropyl β -D-thiogalactopyranoside (IPTG), Luria-Bertani (LB) broth, tryptone, thiamine HCl, and yeast extract were purchased from Hi Media Laboratories, India. Potassium chloride, magnesium sulfate heptahydrate, ammonia, sodium hexameta phosphate, diammonium hydrogen phosphate, potassium dihydrogen phosphate, glycerol, methanol, acetic acid, formaldehyde, silver nitrate, calcium carbonate, and phosphoric acid, hydrochloric acid, sodium chloride, sodium acetate tri hydrate, sodium hydroxide, calcium chloride dihydrate, glacial acetic acid, tri sodium citrate dihydrate, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dihydrate, and trifluoroacetic acid (TFA) were purchased from Merck Millipore Ltd. Ethanol was purchased from SDFCL, India. Tris base, ethylene diamine tetra acetic acid potassium salt, and urea were procured from Sisco Research Laboratories, India. Antifoam was purchased from Sigma Aldrich, USA. All of the chemicals stated above were of preparative grade.

Acrylamide, bis-acrylamide, Tetramethylethylenediamine (TEMED), ammonium persulfate, tricine, and sodium dodecyl sulfate (SDS), Tricine were procured from Sigma and were of analytical grade. Alpha-chymotrypsin (3x crystallized) was purchased from MP Biomedicals, UK. Analytical grade acetonitrile was procured from SDFCL, India.

Bio-Rad Tetra gel assembly, Gel Doc, from Bio-Rad, Akta Avant from GE Life Sciences, and Agilent 1260 Series High Performance Liquid Chromatography system from Agilent Inc. were used in this investigation.

Cloning and expression of rLTNF concatemer

E. coli strain BL21 (DE3) was used as a host for rLTNF production. The rLTNF gene (*Snk8*) was cloned in the *E. coli* expression vector pET29a (Novagen, USA) in the restriction sites NdeI and XhoI, and with a C-terminal 6x-His tag as described previously.²¹ *E. coli* was maintained on LB agar plates and stored at 4°C. The production of rLTNF concatemer protein (molecular weight of 11.4 kDa) was induced at 35° C with 1 mM IPTG.

Production of rLTNF concatemer in bioreactor

Medium for preparation of the seed culture consisted of 0.5% glucose, 0.5% yeast extract, 0.5% sodium chloride, 0.06% magnesium sulphate heptahydrate, and 1% tryptone. The medium was sterilized by autoclaving and the glucose solution was autoclaved separately to prevent Maillard reaction and then mixed under aseptic conditions. For preparing the seed culture, a fresh petri plate was used to inoculate a 250 ml Erlenmeyer flask containing 50 ml of culture medium and the culture was incubated at 30° C for 10 h at 180 rpm in an orbital shaker (Orbitek, Scigenics Biotech, India).

All experiments were performed in a 1.3 L bioreactor (Eppendorf, USA) with an initial volume of 0.3 liter and connected to a gas analyser (BlueSens, Germany). The dissolved oxygen concentration was controlled at 30% of saturation, first by changing the agitation speed between 300 and 900 rpm, after which the supplied air was enriched with pure oxygen

up to 20%. The total gas flow rate was restricted between 0.5 and 1.5 vvm using a mass flow meter. The pH was controlled at $\text{pH } 7.0 \pm 0.2$ by adding 6 N ammonia and 3 N phosphoric acid, and the temperature was maintained at 35°C for batch and fed batch mode of operation. Biocommand software (Eppendorf, USA) was used to monitor and control the experimental apparatus. The substrate feed flow rate was varied using a peristaltic pump (Masterflex® L/S Model No. 7528-30). Batch medium contained 0.6% yeast extract and 20 g/L of initial glucose concentration. The airflow rate during the batch phase was supplied at a constant rate of 0.5 vvm. The glycerol feed concentration used in fed batch mode was 200 g/L. Glycerol was continuously fed to the bioreactor to increase the biomass and exponential feeding strategy was used to maintain specific growth rate of 0.3hr^{-1} . Protein was induced using 1 mM IPTG for 8 hours. The media composition is presented in Table 1.

Theoretical prediction of rLTNF concatemer and rLTNF monomer properties

ProtParam, a web based interactive tool (<http://web.expasy.org/protparam/protparam-doc.html>), was used to compute the physicochemical properties of rLTNF concatemer from its primary sequence. The parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). The tool computes parameters based on algorithms which are discussed in detail in the literature.²²

Solubilization of inclusion bodies (IBs) and purification of the rLTNF concatemer

Upon completion of fermentation, the broth was harvested to obtain cells by centrifugation. The cells were washed with 0.9% NaCl (10 ml per a gram of cells) and the washed cells were collected into the 500 ml beaker using lysis buffer (100 mM Tris, 5 mM EDTA, 100 mM NaCl, pH 8). The cells were lysed using an ultrasonicator (Oscar Ultrasonics, India). Thirty cycles were performed to lyse the cells (30 sec ON/OFF). Cells were centrifuged at 7000 RPM (11.5 G), 4°C , for 15 min to obtain IBs. Solubilization of IBs was carried out using 100 mM Tris and 6 M urea, pH 8, at a ratio of 10 ml buffer to 1 g of IBs. The IBs were kept for stirring at room temperature for 2 hrs. Solubilized IBs (SIB) were adjusted to pH 3 using 5.5 M HCl to remove some of the process related impurities. The concentration of rLTNF concatemer was estimated using HPLC. The rLTNF concatemer was purified using Hitrap SP-FF (7 x 25 mm) from GE Life Sciences, Sweden. The column was equilibrated with 5 column volumes of buffer A (100 mM sodium acetate, 4 M urea, pH 4.0) and then the protein was loaded followed by washing with 4 column volumes of 1 M NaCl. Step elution was performed using 4 column volumes of buffer B (50 mM Tris HCl, 4 M urea, pH 8.0) and cleaning in place of the column was achieved by using 0.2 M sodium hydroxide. Flow rate of the column was 1 ml/min. Elution was monitored at UV 280 nm. The purification experiments were performed on ÄKTA Avant chromatography system (GE Healthcare, Uppsala, Sweden) equipped with pumps, online indicators (pressure, A_{280} , conductivity, pH) and automated fraction collector with cooling facility. Monitoring and control were performed using UNICORN 6.3 software (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Cleavage of the rLTNF concatemer

The elution pool from the capture step was diluted 2x with the enzyme reaction buffer (100mM Tris-HCl, pH 8). To this diluted solution, calcium chloride (10 mM final

concentration) in Tris buffer was added followed by 5 BTEE units of alpha-chymotrypsin per mg of protein. The reaction mixture was incubated at room temperature for 2 hours and reaction was stopped using glacial acetic acid by slowly adjusting the pH to 4.

Purification of rLTNF monomer

rLTNF monomer was obtained and purified using Kromasil reverse phase C8 column (4.6x250 mm, 110 Å, 10µ). Monomer was separated using buffer A (10 mM trisodium citrate, pH 6) and buffer B (acetonitrile). Column was equilibrated using 5 column volumes of 95% buffer A and 5% buffer B and then sample was loaded followed by washing using 4 column volumes of 95% buffer A and 5% buffer B. Elution was performed using 20 column volumes of buffer B linearly increasing from 5% to 28% and regeneration was carried out using 90% buffer B. Flow rate of the column was 1 ml/min and elution was monitored at UV 220 nm. During elution, fractions were collected, analysed and pooled. The elution pool was freeze dried using a lyophilizer with option of handling organic solvents.

Analytical methods

For biomass measurement, 5 ml of culture broth was harvested at hourly intervals. Cell growth was monitored by measuring optical density at 600 nm using a spectrophotometer (Molecular Devices, USA). For dry cell weight measurement, 1 ml of sample was centrifuged at 12000 *g* for 5 min in pre-weighed centrifuge tubes. Then cell pellet was washed with distilled water and dried at 60 °C for 24 hours. Glucose and acetate concentration were analyzed using Hi-Plex H column (Agilent Technologies, USA). Protein expression was analyzed by Tricine-SDS-PAGE using 12% polyacrylamide gel.²³ Quantitative estimation of rLTNF multimer was determined by a RP-HPLC method. Proteins were resolved on a TSKgel Protein C4-300 (4.6x150 mm, 3µ particle size) column from Tosoh, Japan, using a 40–100% acetonitrile gradient with water in the presence of 0.1% TFA at 40 °C. Elution was monitored at 220 nm. Samples during cleavage reaction were analyzed by using an Acquity UHPLC BEH C18 column (2.1x50 mm, 1.7µ particle size) from Waters Corporation, USA. The column was equilibrated with 100% buffer A (0.1% TFA with water) for 10 min followed by injection of the sample. Elution was performed using 0–55% buffer B (0.1% TFA with ACN) over 60 min. The column was operated at the flow rate of 0.2 ml/min. The analysis was performed at 40 °C and monitored at 220 nm. Both the methods were developed using ultimate 3000 HPLC from Thermo Fisher Scientific, USA.

Techno-economic analysis of the process

Figure 8 presents an illustration of the proposed process. Fermentation was performed in aerobic conditions to produce the concatemer in the form of cytoplasmic inclusion bodies. The harvested cells from the broth were subjected to lysis for releasing IBs, which were recovered by centrifugation. Solubilization of IBs was performed in the presence of denaturant. Concatemer was captured using CEX chromatography. Further purification involved cleavage of concatemer, RP HPLC purification, and freeze drying. The process was able to produce 4.15 g of peptide per batch at 1L scale of fermentation. Using this template, the process for 10 kg per batch was simulated.

The annual capacity of the plant was estimated for the current process to be 3050 kg of purified rLTNF per year (Supplementary Figure 1). A brief description of the plant and its operation is described here. Fermenter (FR-101) is fed with media, sterile air, water, and inoculum. Total run time of step is approximately 24 h with temperature maintained at 37°C. The broth at the fermentation is transferred to storage tank (V-102). A disc stack centrifuge (DS-101) is used to harvest the cells from the broth. The centrifuge reduces liquid volume of the process stream and removes media components. Cell homogenizer (HG-101) was used to break the cells and this results in releasing of inclusion bodies along with cell debris. The temperature was maintained at 10 °C. After lysis, the centrifuge was used to separate cell debris from inclusion bodies. The solubilization was performed in vessel (V-101) at room temperature following the depth filtration to remove any particulate matter before loading onto the CEX column (C101). The elution pool from CEX and the cleavage reaction were carried out in (R-101). The reaction end was purified using RP HPLC (C-102). The final product was obtained in powder form after freeze drying using freeze drier (FDR-101). The overall recovery of primary recovery stages was 95%. Overall a total of 274 kg of IBs were recovered from 942.69 kg of biomass which were further processed to obtain 10 kg of purified rLTNF-11 per batch. SuperPro Designer V9 (Intelligen Inc., Scotch Plains, NJ) was used to perform process simulations, mass and energy balances, and cost analysis. Data from lab scale process was used as template for simulating the plant for manufacturing 10 Kg of rLTNF. The software uses material balance for each unit operation to adjust sizing of equipment during scale up (http://www.intelligen.com/superpro_features.html).

Assuming the demand of 3 ton of final product per year, scheduling of plant equipments, operation cost were determined. The costs of equipment and indirect costs were inbuilt in the software. The costs of raw materials (chemicals and reagents) were collected from local vendors. Economic analysis was performed in US Dollars (\$).

RESULTS AND DISCUSSION

Recombinant expression of rLTNF

E. coli BL21 (DE3) was transformed with a plasmid construct containing the rLTNF peptide. Strong expression of rLTNF peptide was observed in the Tricine gel (Figure 1) in shake flask experiments. The band in lane 5, corresponding to 11.4 kDa, represents the rLTNF concatemer expressed in the form of IB.

As it is well established that T7 system exhibits leaky expression, it can be inferred from the data (Figure 1 Lane 4) that clone used in the current work also showed leaky expression during uninduced condition.^{24, 25}

After confirming expression in shake flasks, a batch fermentation of *E. coli* BL21 (DE3) was performed with 20 g/L of glucose as carbon source. Once batch was completed at the onset of rise in dissolved oxygen (Figure 2a), 200 g/L of glycerol feeding was used. Glycerol feed was added to introduce a carbon source so as to achieve higher cell density. Feed rate was increased exponentially to maintain a specific growth rate of 0.3 h⁻¹ at 35 °C (Figure 2b).

Induction was performed at 11.75 g/L dry cell weight (DCW) using 1 mM IPTG and protein production was performed for 8 hours. Biomass yields of up to 32.30 g/L were obtained and higher concentrations of glycerol were found to trigger excess acetate production (Figure 2b). Acetate accumulation was found to be 3.23 g/L due to lower TCA cycle activity coupled with altered transcription levels of genes associated with glycerol and acetate metabolism.^{25, 26} Metabolism of the *E.coli* B strain is limited in its capacity to control acetate formation at limiting dissolved oxygen.²⁶ However, BL21 (DE3) can utilize acetate and reduce it to negligible amounts at the end of the fermentation.

Product was accumulated as inclusion bodies with product concentrations reaching up to 4 g/L (Figure 2c). The results indicate that higher biomass may be achieved if continuous feed of carbon source and oxygen are made available. An HPLC profile of the product obtained from the solubilized IBs is shown in Figure 3a, and the peak corresponding to 4 min retention time (RT) was confirmed as the rLTNF concatemer. The molecular mass of 11.4 kDa was found to be consistent with the isotopic mass of the rLTNF concatemer (Figure 4a).

Isolation and purification of the rLTNF concatemer

It is evident from the data shown in Figure 1 that the rLTNF concatemer is expressed as cytoplasmic IBs. The harvested cells are lysed to release the IBs along with intracellular components. IBs are then recovered by centrifugation. However, contaminants such as cell wall components, host cell DNA (HCDNA), host cell proteins (HCP), and other soluble host components adhere to the IBs. There are methods for efficient removal of these impurities, such as washing with detergents like deoxy cholate, Triton X-100, and mild denaturing agents (urea, guanidine-HCl).^{27–31}

Although these methods can be quite effective for obtaining pure IBs in laboratory experiments, the use of additional chemicals followed by repetitive washing and centrifugation steps add to the operation costs at industrial scale. Therefore these steps need to be carefully selected and optimized.³⁰ In this paper, recovered IBs prior to washing were solubilized in Tris Buffer with 6 M Urea. This facilitated the solubilization of concatemer along with the impurities. The resulted mixture exhibited high viscosity, likely due to the presence of HCDNA, HCP, and other contaminants. Adjusting the pH to 3 with HCl resulted in precipitation of large amount of impurities with negligible loss (<0.25%) of any product as analyzed by HPLC. At acidic pH, the majority of impurities are known to exclude the solvent and hence result in floc formation, easily removable by centrifugation.³²

The concentration of concatemer in SIB, as determined by HPLC was found to be 4.15 g/L. The physicochemical properties (molecular weight, pI, hydrophobicity) of the concatemer were taken into consideration for designing the downstream process. Various parameters determined by ProtParam by using primary sequence of the protein.. The higher absorption coefficient compared standard protein such as bovine serum albumin was attributed to the fact that the amino acid composition in the primary sequence of the protein includes 9.2% tryptophan. The instability index of 48.58 classifies the protein as unstable.³³ Other properties include isoelectric point (6.56), molecular weight (11.404 kDa), and hydrophobicity index (−0.387).

Capture step plays an important role in purification. It removes a significant amount of process related impurities such as HCP, HCDNA, and pigments. Many researchers have reported use of Nickel-Nitrilotriacetate (Ni-NTA) based chromatography as capture step.^{34, 35} This mode of chromatography purifies the protein to more than 95% with a product recovery of 90% in a single step. However, it faces many limitations and disadvantages such as limited binding capacity, nickel leaching (which limits column lifetime), nonspecific binding, metal catalyzed oxidations of amino acids, and protein cleavage.^{36–38} On the contrary, ion exchange chromatography (IEX) offers several advantages such as high binding capacity and ease of scale up. Taking into consideration the resulting increased titer, ease of operation and other advantages, IEX was preferred over IMAC for product capture in current case.

The theoretical pI of the rLTNF concatemer was 6.56. Therefore cation exchange chromatography was chosen as initial choice for the process. Initial experiments with SP Sepharose FF resin at pH 4, 5, and 6 using salt gradient-based elution revealed that the protein bound strongly to the resin and eluted in the regeneration step (0.2 M NaOH). The strong binding may be due to acidic amino acid residues constituting around 9.2% of the protein. Therefore, the effect of pH on protein elution was investigated. Protein was bound at pH 4 and eluted using a pH gradient from 4 to 10 using buffer consisting of citrate, sodium dihydrogen phosphate, tricine, and glycine (20 mM each).³⁹ The desired product was eluted at pH 7 (just above the pI of the protein). Based on the binding and elution characteristics of the protein, it was decided to bind the protein onto the column at pH 4 (20 mM sodium acetate) and loosely bound impurities were washed by 1 M NaCl followed by product elution at pH 8 (20 mM Tris-HCl) using a step gradient (Figure 5). It should be stated that the eluted protein was turbid due to precipitation, most likely because the instability index of the protein is high and is classified as unstable. Moreover, hydrophobic amino acids (34.7%) might have been exposed to water, causing the protein to precipitate. Urea, due to its hydrogen bonding ability, is known to enhance solubility of proteins. The addition of 4 M urea to the equilibration and elution buffers aided greatly in reducing precipitation. This step was used to remove impurities, obtain concentrated protein, and to make the elution pool suitable for enzymatic cleavage reaction. Dynamic binding capacity (DBC) was determined to be 140 mg/ml of resin and step yield was 97±1%.

Cleavage of rLTNF

As described earlier, the rLTNF peptide was expressed as tandem repeats of monomer along with His tag (referred to as concatemer). To obtain the peptide in an active form, cleavage at the C terminal of tryptophan residue was required. There are numerous reports on chemical cleavage of tryptophan at its C terminal side by using cyanogen bromide, O-Iodoso benzoic acid, N-bromosuccinimide, and N-chlorosuccinimide.^{40–43} However, most of these methods suffer from poor yield, poor specificity, and/or propensity for side reactions. Experiments were performed with N-bromosuccinimide and CNBR with limited success (data not shown). Moreover, rLTNF has one methionine residue at 4th position which is prone to oxidation under these reaction conditions, therefore it was decided to perform the enzymatic cleavage with alpha-chymotrypsin, a serine protease which cleaves the peptide bond at the C-

terminal of tryptophan. It is also an endoprotease with an optimum pH of 8 and requires calcium chloride (CaCl_2) for its enzymatic activity.

It is well known that the enzyme is stable in urea up to 2 M concentration.^{40, 41} The elution pool obtained from the capture step was in 20 mM Tris- HCl pH 8 with 4 M urea and the concatemer concentration was between 10–12 mg/ml. Hence, dilution was necessary for maintaining the stability of the enzyme during the reaction conditions. The concatemer was found to be completely digested in 45 minutes (Figure 6). During the course of reaction many impurities were generated either due to nonspecific cleavages or due to reaction with HCPs remaining in CEX chromatography elution pool. LC-MS results confirmed that the peak eluting at 36.6 min (Figure 3b) was rLTNF with a mass of 1268 (Figure 4b). The purity of reaction mixture was around 50–60%, indicating the need for further purification.

RP-HPLC has been the traditional workhorse for purification of peptides and small proteins.^{42–44} Based on the analytical profile, it was decided to use a C8 column instead of C18. Among the different buffers screened (acetate pH 4.5, citrate pH 5.5, 25 mM acetic acid), best resolution was obtained with citrate buffer at pH 6. The theoretical pI of rLTNF-11 is 5.56 and as the buffer pH approaches pI of the peptide, selectivity increases due to increase in hydrophobic interaction. Moreover, citrate by its nature induces the salting out effect. Both of these factors resulted in higher resolution compared to other buffers.

Buffers with pH greater than 6 were not examined since stability of the resin has been known to decrease under these conditions. Preliminary experiments with citrate buffer and acetonitrile as eluent confirmed that the elution window lay between 19–21% of buffer B (Figure 7). The gradient was optimized for better resolution. The rLTNF-11 peak was fractionated and pooled to obtain purified rLTNF-11. The step yield of RP HPLC was 96–98% with a purity of more than 98%. The overlay of analytical chromatograms for load and elution pool is shown in Figure 3c. The pool material was freeze dried to obtain the final product in a powder form.

Techno-economic analysis of the process

The overall material balance is as shown in the Figure Supplementary Figure 2. The overall in/out balance was 27824 Kg/batch. Costs related to cleaning of equipment and facility maintenance were neglected. As per our calculations, about 3.8 ton of acetonitrile was used per batch (highest cost among the other raw materials). Supplementary Figure 3 shows the Gantt chart generated by the software. Single batch operates for 64 hours and produces 10 kg of rLTNF and if manufactured throughout the year, a total of 3 ton of main product can be produced per year.

The tables are part of reports generated during economic evaluation by the SuperPro Designer. Supplementary Table 1 shows the list of equipment that need to be procured with preliminary specifications and unit cost. Considering the base case (3 ton/year), the total cost of different equipment, vessels, and reactors that need to be purchased was around \$9.3 million. Among the equipment, the freeze dryer was the most expensive (30% of total cost). Others major contributors were the process chromatography columns (13%) and the

centrifuge (15%). The total fixed capital investments are as shown in Supplementary Table 2 (\$54.2 million).

Raw materials place an important role for yearly operation for successful main product generation in expected quantity. Supplementary Table 3 shows raw material consumption per year. Amongst the raw materials, acetonitrile contributes most (around 57.3%) followed by media (21.7%) and water (15.2%). It is observed that enzyme alpha chymotrypsin contributes to only 0.01% of the total raw material costs, likely due to lower cost of the chemical and lower utilization per gram of protein. Moreover, with the advent of improvements in the purification process, enzymes with high specific activity are generally used in very minute quantities even at large scale resulting in reduction of cost of this raw material. The process uses complex media which contributes to around 20% of cost. Improving the fermentation process by utilizing chemically defined media might reduce the cost and making it a more cost effective process. Consumable costs are enlisted in Supplementary Table 4, of which chromatography resins (RP HPLC resin cost is represented in INX resin) contribute the most.

Supplementary Table 5 shows the annual operating cost breakdown which is around \$20.3 million. Facility dependent cost contributes to around 50% followed by raw materials (20%) and consumables (20%) which is shown further in the form of pie chart in Figure 9.

In summary, it can be expected that the final manufacturing cost for rLTNF will be in the range of \$5 to \$6 per gram.

CONCLUSIONS

The process for manufacturing the recombinant rLTNF has been developed with overall recovery of 78–80%. A manufacturing plant capable of producing 10 Kg of rLTNF per batch was simulated and techno economic analysis performed. The production cost of rLTNF was estimated to be \$5–6 per gm (or \$0.09 per 15 mg dose), significantly lower in cost to that of currently available antivenom in the market (approximately \$6 per dose). The overall study proves that rLTNF can be produced cost effectively through recombinant route.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tricine gel (12%) electrophoresis of shake flask samples. Lane 1: Marker, Lane 2: Uninduced cell lysis supernatant, Lane 3: Induced cell lysis supernatant, Lane 4: Uninduced solubilized inclusion bodies, lane 5: Induced solubilized inclusion bodies

Figure 2.

a) Fermentation profile of rLTNF concatemer production b) Biomass profile of *E. coli* BL21 (DE3) measured in dry cell weight (DCW), Acetate and optical density (OD), c) Product profile of *E. Coli* BL21 (DE3) using HPLC analysis.

Figure 3.
Representative analytical RP- HPLC chromatogram of (a) SIB, (b) Enzyme reaction end, and (c) Overlay of analytical chromatograms of RP HPLC load and elution pool.

Figure 4.

a) Deconvoluted mass spectra of rLTNF multimer b) Mass spectra of rLTNF monomer after enzyme reaction.

Figure 5.
Chromatogram of preparative CEX chromatography

Figure 6.
Profile of cleavage reaction of concatemer

Figure 7.
Preparative RP-HPLC chromatogram of purification of LTNF from reaction end

Figure 8.
Overview of manufacturing process for production of LTNF

Figure 9.
Annual operating cost breakdown (\$20.31 million)

Table 1

Media composition used for production of rLTNF concatemer in bioreactor

Nutrient g.L ⁻¹	Batch	Fed batch
Glucose	20.0	--
Yeast extract	4.80	1.80
Magnesium sulphate hepta hydrate	0.49	2.74
Thiamine HCl	0.16	0.06
Sodium hexameta phosphate	12.23	--
Diammonium hydrogen phosphate	1.84	0.78
Potassium chloride	0.41	--
Potassium dihydrogen phosphate	--	2.62
Glycerol	--	200
Antifoam A (mL.L ⁻¹)	1.00	1.00
Kanamycin (µg.mL ⁻¹)	50.00	50.00
IPTG	--	0.24