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Influence of Cell Disruption Methods on the Recovery and Immunogenicity of a Fusion Protein for a Therapeutic Cancer Vaccine Against HPV

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Abstract

Two cell disruption methods, mechanical and chemical, were applied for the recovery of a fusion protein named CIGB 550-E7, expressed on *Escherichia coli* grown in defined saline media. A comparison of the methods was done, and various operating parameters for each technique were optimized to obtain the maximum disruption efficiency and CIGB 550-E7 protein release. The mechanical disruption's yield and recovery were 1.24 and 1.37 times higher than those obtained with chemical disruption.

Modified conditions were assayed for the CIGB 550-E7 obtained by chemically defined media using the mechanical and chemical cell disruption methods. The processes we developed allowed us to obtain an active pharmaceutical ingredient that fulfills the requirements stipulated by the regulatory authorities in terms of purity and lipopolysaccharide contaminants. In addition, the CIGB 550-E7 obtained from both methods showed similar biological activity so that either method could be used. Finally, a cost/benefit relationship (CBR) analysis was done for both disruption methods, and the CBR value for mechanical cell disruption demonstrated that this was the most feasible choice.

Introduction

Cervical cancer is the second most common cancer in women throughout the world. In the 270,000 deaths caused by cervical cancer annually, more than 85% occur in developing countries.^[1] Preventive programs have been established in developed countries, making most precancerous lesions identifiable at earlier stages when they can be treated easily. Early treatment prevents 80% of

cervical cancers in these countries. However, the limited access to effective screening tests means that the disease is often not identified until it is further advanced and symptoms have developed. In addition, the prospects for treatment of such a late-stage disease may be poor, resulting in a higher rate of death from cervical cancer in these countries. Most cervical cancers can be attributable to human papilloma virus (HPV) infection, and there are over 150 related types of HPV virus. Most cases of cervical cancer and its precursor lesions are related to infection by high-risk HPV serotypes, especially HPV-16 and HPV-18.^[2,3]

There are currently three vaccines, Gardasil® (or Silgard®) and Gardasil 9 (Merck & Co.) and Cervarix® (GlaxoSmithKline), which protect against certain strains including HPV-16 and HPV-18, the serotypes responsible for 70% of cervical cancers. These vaccines are recommended prior to HPV exposure since they cannot treat HPV infection or HPV-associated diseases such as cancer. Therefore, it has been necessary to develop a therapeutic vaccine for the treatment of established lesions and cancer associated with HPV infection.

In a previous paper, Granadillo *et al.*^[4] described the design of a fusion protein named CIGB 550-E7, which is comprised of a cell-penetrating and immune-stimulatory peptide that is linked to the E7 antigen of HPV-16, as the main ingredient for a therapeutic vaccine against cancer caused by HPV.^[4] The results have shown that this recombinant protein is expressed in *Escherichia coli* that has been transformed with the pPEPE7M-7K plasmid. Expression levels reached 18% in a complex medium, and the yield was 38 mg of CIGB 550-E7/L of culture that was induced at the end of the fermentation stage. In animals, biological properties demonstrated that this fusion protein is immunogenic with anti-tumor activity, making it a reasonable candidate for clinical trials in humans.^[4] However, this vaccine candidate does not meet the requirements of regulatory authorities for human use. Therefore, some changes need to be carried out before we reach the clinical trial phases.

Designing a biopharmaceutical process intended for the

manufacture of a vaccine is a complicated effort that must take into account numerous factors such as: safety, economy, scale, environment, product lifecycle, administration, good manufacturing practices, and others. The process design should address robustness to assure that the product will repeatedly provide the expected yield while meeting the defined specifications. To attain a more consistent product, defined mineral salt media are generally preferred. These media attain more consistent expression levels, allow easier process control and monitoring, and simplify downstream recovery of the target protein.

Another factor that complicates the recovery process is the fact that the product is retained inside a microbial cell, and in this case, the protein must be released from *E. coli*.^[5] At industrial-scale, high-pressure homogenization and ball mills are two widely used procedures for mechanical cell disruption.^[5] However, these mechanical methods have certain drawbacks since they break the cells completely, and all of the intracellular materials are released. Then the target product must be separated from a complex mixture of proteins, nucleic acids, and cell wall fragments. In addition, released bacterial nucleic acids can increase the viscosity of the solution and may complicate subsequent clarification steps.

Another way to disrupt cells is to use chemical methods

to extract intracellular components from microorganisms by permeating the outer wall barriers. This can be achieved with organic solvents, antibiotics, thionins, surfactants, chaotropic agents, and chelates. As rupture is the first recovery process, it impacts all downstream steps. For this reason, optimization of this step is important. Mechanical and chemical disruption methods are described in this research paper.

In this article, immunogenicity and total recovery measurements for CIGB 550-E7 were performed to evaluate the influence of chemical and mechanical cell disruption. We evaluated chemical disruption first to avoid extensive cell fragmentation and decrease the release of contaminants which would impact the downstream process performance. In these experiments, we examined the potential of pH, urea, and Triton X-100 to release the target protein.

For mechanical disruption, we evaluated the influence of a number of disruption cycles, cell concentrations, and pH on the disruption efficiency. Screening and response surface experimental designs were used to evaluate the overall effects.

Finally, the optimum conditions were further evaluated in the downstream process for the recovery of the CIGB 550-E7 from *E. coli* grown in defined mineral salt media.

Materials and Methods

Materials

Chemicals were purchased from [Merck KGaA](#) and [Sigma-Aldrich Co.](#) All solutions were prepared using water for injection (WFI) produced by a pure steam generator located at the Center for Genetic Engineering and Biotechnology (CIGB) in Havana, Cuba.

Bacterial Growth

CIGB 550-E7 is a fusion protein containing a cell-penetrating and immune-stimulatory peptide corresponding to residues 32–51 of the *Limulus polyphemus* protein. This peptide is linked to the HPV-16 E7 antigen (LALF32-51-E7), and is expressed in *E. coli* BL21 (DE3). The cells harboring the pPEPE7M-7K plasmid were grown in 50 mL MDGK broth at pH 6.5. This medium is composed of 3.55 g/L Na₂HPO₄, 3.4 g/L KH₂PO₄, 2.7 g/L NH₄Cl, 0.5 g/L MgSO₄·7H₂O, 5 g/L glucose, 0.3 g/L Na₃C₆H₅O₇·2H₂O, 1 mL/L trace salts 1000X, 0.05 g/L kanamycin, and 2.5 g/L C₄H₇NO₄. After 24 hours at 28 °C on a rotator shaker at 200 rpm, a sufficient number of cells were produced to inoculate a 5 L fermenter ([B.E. Marubishi](#)). The highly defined medium in the fermenter consisted of 3.55 g/L Na₂HPO₄, 3.4 g/L KH₂PO₄, 2.7 g/L NH₄Cl, 2.5 g/L aspartic acid, 0.3 g/L citric acid, 0.5 g/L MgSO₄·7H₂O, 5 g/L glucose, 10 g/L glycerol, 0.01 mg/L thiamine, 0.05 g/L kanamycin, 1 mL/L trace salts 1000X, 94.5 mg/L L-tyrosine, 13.5 mg/L L-phenylalanine, 66 mg/L L-isoleucine, and 157 mg/L L-serine. The fermenter parameters were pH 6.5, 35 °C, 700 rpm, and 50% dissolved

oxygen. This system was operated in batch mode for 24 hours, and then the cells were harvested by centrifugation at 3000 rpm for 30 minutes at 4 °C.

Mechanical Disruption Experiments

Experimental Screening Design for Mechanical Disruption

A screening experimental design was used to identify the influence of cell concentration, pH, and number of passes on rupture efficiency. Independent variable levels are shown in **Table 1**, and values were selected according to Granadillo *et al.* and Duong.^[4,5]

Rupture efficiency was defined as Co/C where Co was the total protein concentration at each pass, and C was the total concentration at the last pass. Typically, five passes are required to break 100% of the *E. coli* cells.

Experiments were performed with 5 g of biomass at the required pH with 5 mmol/L NaH₂PO₄, 30 mmol/L NaCl, and either pH 8.0 with 81.1 mmol/L NaHCO₃, or 19 mmol/L

TABLE 1. Independent variables levels for screening design for mechanical disruption.

Factor	Low	High
Cell concentration % (w/v)	10.0	60.0
pH	8.0	10.6
Number of passes	2.0	5.0

Na₂CO₃ at pH 10.6. The resulting biomass material was homogenized, and for each set of conditions, five cycles were assayed in a high-pressure homogenizer (French-type press). Pressure was maintained between 118–147 MPa. Samples were taken after each pass and centrifuged at 10,000 rpm for 30 minutes at 4 °C. Each sample was analyzed for total protein using a micro-Coomassie colorimetric method^[6] as well as electrophoresis in polyacrylamide gel. Rupture curves were prepared using the procedure described by Duong.^[5]

Response Surface Experimental Design for Mechanical Disruption

Using the results obtained (described above), a response surface experimental design was conducted. A step ± 5 was considered for cell concentration and ± 1 for pH. Total protein and purity of the target protein were both quantified to determine the yield of the rupture stage in mg of CIGB 550-E7/g of biomass. Independent variable levels are shown in **Table 2**.

The procedure followed during the experiments involved homogenizing 2 g of biomass and the required rupture solution according to **Table 2**, which was 5 mmol/L NaH₂PO₄ and 30 mmol/L NaCl, at pH 7.0, as well as 5 mmol/L NaH₂PO₄ and 30 mmol/L NaCl at pH 8.0, or 5 mmol/L Tris and 30 mmol/L NaCl at pH 9.0. The resulting mixture received three cycles (as suggested earlier) in a high-pressure homogenizer (French-type press) while maintaining the pressure between 118–147 MPa. The resulting material was centrifuged in a refrigerated [Hitachi model SCR7B](#) for 30 minutes at 4 °C at 10,000 rpm. Samples were analyzed for total protein using a micro-Coomassie colorimetric method and electrophoresis in polyacrylamide gel.

Chemical Cell Disruption

Screening Experimental Design for Chemical Disruption

An alkaline lysis, the use of the chaotropic agent urea, and Triton X-100 detergent were studied. The dependent

TABLE 2. Independent variable levels for a response surface design for mechanical disruption.

Factor	Low	Center Point	High
Cell concentration % (w/v)	5.0	10.0	15.0
pH	7.0	8.0	9.0

TABLE 3. Independent variables for a screening experimental design for chemical disruption.

Factor	Low	High
pH	8.0	10.6
Urea (mol/L)	0	1.0
Triton X-100 (%)	0	1.0
t (min)	30.0	60.0

variable was Co/C, and the levels for each variable are summarized in **Table 3**. Rupture efficiency was evaluated as the relation between total protein concentration in the supernatant achieved with chemical disruption versus mechanical disruption.

The procedure involved 20 g of biomass. It was washed with 200 mL of WFI, and then centrifuged at 3,000 rpm for 30 minutes at 4 °C in a refrigerated centrifuge (Hitachi, SCR7B). The solid was homogenized with 200 mL of rupture solution consisting of 5 mmol/L NaH₂PO₄, and 30 mmol/L NaCl at pH 8.0. For each experiment, 10 mL were mixed with either urea or Triton X-100, and the pH, as indicated for each variable (as described in **Table 3**). The resulting material was centrifuged at 10,000 rpm for 30 minutes at 4 °C. Samples were analyzed for total protein using a micro-Coomassie colorimetric method and electrophoresis in polyacrylamide gel.

Response Surface Experimental Design for Chemical Disruption

Independent variables were selected according to the results of a screening design, and levels are shown in **Table 4**. The dependent variable was the quantity of released protein (mg CIGB 550-E7).

The procedure followed during the experiments was for 20 g of biomass. It was washed with 200 mL of WFI and the precipitate from the centrifugation step was homogenized with 200 mL of rupture solution (described earlier). Ten mL were used for each sample, the required components were added, and the pH was adjusted with 500 mmol/L NaOH, as is shown in **Table 4**. Samples were incubated with agitation for one hour and the resulting material was centrifuged at 10,000 rpm for 30 minutes at 4 °C. Samples were analyzed for total protein using a micro-Coomassie colorimetric method and electrophoresis in polyacrylamide gel.

CIGB 550-E7 Purification

Cells were harvested by centrifugation and disrupted using the optimum conditions obtained for mechanical or chemical cell disruption. Inclusion bodies recovered from cell lysates and containing the CIGB 550-E7 were centrifuged at 10,000 rpm for 30 minutes at 4 °C. The pellet fraction was washed (pre-extracted) with 5 mmol/L NaH₂PO₄, 30 mmol/L NaCl, 1 mol/L urea, 10 mmol/L betamercaptoethanol, and 1% Triton X-100, at pH 8.0 to remove heavily contaminated *E. coli* cell walls and outer membrane components. Solubilization of the CIGB 550-E7 protein was achieved with a buffer consisting of 81.1 mmol/L Na₂CO₃, 19 mmol/L NaHCO₃, 6 mol/L

TABLE 4. Independent variables for a response surface design for chemical disruption.

Factor	Low	Center Point	High
pH	8.0	10.0	11.0
Triton X-100 (%)	0.2	0.5	1.0

urea, 10 mmol/L betamercaptoethanol, and 0.01% Tween 80, at pH 10.6. Purification was carried out on HIS-Select® nickel affinity gel (SAFC) using standard procedures. Finally, after size exclusion chromatography using a Sephadex G-25 matrix (GE Healthcare), the active pharmaceutical ingredient (API) was obtained with 10 mmol/L Tris, 150 mmol/L NaCl, and 0.01% Tween 80 at pH 8.0.

Biological Activity Assay

Mice

Female C57BL/6 mice (8–10 weeks old), were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) and housed in the animal care unit. All experiments were conducted in accordance with institutional guidelines for animal care.

Adjuvant and Immunizations

Aluminum salt or alum, Al(OH)₃, was provided by CIGB and used at a working concentration of 0.6 mg Al³⁺/mouse. The vaccine formulations were prepared the day of injection

with each formulation containing 0.12 mg of CIGB 550-E7 that was obtained by physical and chemical cell disruption methods and adjuvanted with 0.6 mg alum. For the placebo group, the excipient was mixed with 0.6 mg alum. The total injected volume was 0.5 mL/mouse. All immunizations were given subcutaneously in the flank, twice at one-week intervals.

Implantation of TC-1 Tumor

In preparation for mouse implantation, TC-1 cells were cultured until they were approximately 70% confluent, and then harvested with trypsin. Fourteen days after the last immunization, the TC-1 cells were injected subcutaneously in the leg using a 25 gauge needle at 5×10^4 doses in 0.2 mL/mouse. Starting 3–4 days later, the area was observed daily and palpated for the presence of a tumor nodule.^[7]

Statistical Analysis

The statistical significance of the tumor protection was determined using the log-rank test.

Results and Discussion

Determining Operating Conditions for Mechanical Cell Disruption

The influences of the independent variables: cell concentration, pH of the ruptured solution, and the number of passes through the valve assembly with a French-type press were studied with the screening experimental design defined earlier. **Figure 1** shows that cell concentration and its interaction with pH had a positive influence on cell disruption efficiency, while pH had a negative influence, using a 95% trust level with $R^2 = 84.2\%$.

From **Figure 2**, it was concluded that three passes through the valve were enough to obtain 100% disruption efficiency. However, this parameter doesn't significantly

influence the rupture efficiency, as was demonstrated in **Figure 1**. Values higher than 100% were distorted by the total protein determination method. In addition, samples were analyzed by electrophoresis, and the CIGB 550-E7 protein was located in the ruptured precipitate that was corroborated by Western blot (figures not included in this work). These results correspond with those obtained by other researchers, establishing that protein E7 expressed in *E. coli* forms insoluble inclusion bodies that are located in the rupture precipitate.^[8,9]

Optimization of parameters by the conventional method involves changing one independent variable while keeping all others at a fixed level. While this is extremely time-

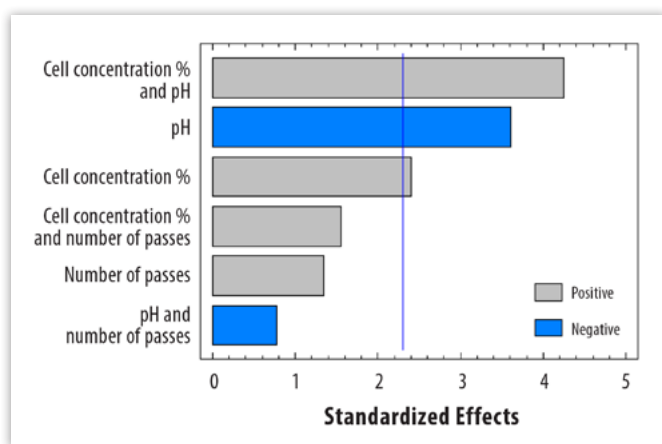


FIGURE 1. Pareto chart for screening the experimental design for mechanical cell disruption. The influence of cell concentration, pH, and number of passes on disruption efficiency are shown.

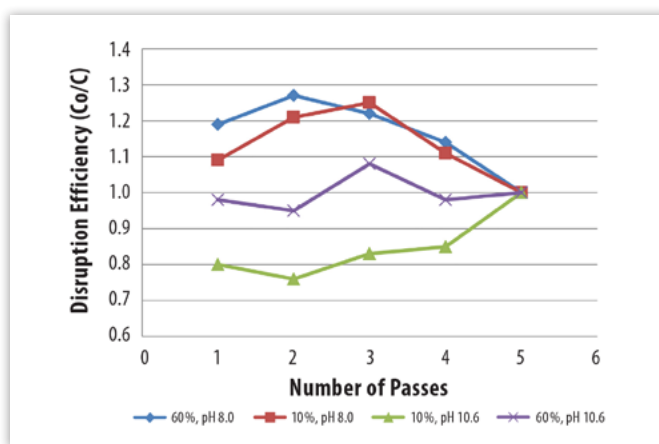


FIGURE 2. Protein release curves obtained at pH 8.0 and 10.6 using 10% and 60% (w/v) of cell concentration.

consuming and expensive for a large number of variables, the response surface methodology (RSM) is one of the strongest tools known to estimate the effect of many factors with a lower number of experiments, and can also be used to refine the optimization of the process. It defines the effects of each variable independently, as well as the contribution of joint effects of variables that cannot be observed by traditional optimization methods. Therefore, starting from the results obtained in the screening design, a new experiment based on a response surface type was developed to establish the rupture conditions for the CIGB 550-E7 fusion protein.

The centerpoint of this design was pH 8.0 and a 10% cell concentration. Independent variable levels are shown in **Table 2**. From the Pareto chart shown in **Figure 3**, pH had a positive influence, and cell concentration² had a negative influence on yield for 95% of confidence with $R^2 = 69.7\%$.

The optimization carried out by the program suggested that a maximum of the protein release yield was at 12.1 mg CIGB 550-E7/g biomass when the pH was 9.0 and cell concentration was 10% w/v. Then three experimental replicas were done in order to confirm this value. Results are shown in **Table 5**.

As the relative error was less than 10%, the conditions were accepted for this pH and cell concentration.

Chemical Rupture as an Alternative to the Mechanical Rupture

One alternative to mechanical disruption is to utilize chemicals which interact with the cell structure in a manner which causes it to become permeable. In this paper, the influence of pH, reaction time, the use of the chaotropic salt urea, and the detergent Triton X-100 on rupture efficiency was studied. A screening experiment was designed, and the independent variable levels are shown in **Table 3**. Disrupted samples were treated following the experimental design results shown earlier.

The effect of each variable studied is shown on the Pareto chart (**Figure 4**), which indicates that pH and Triton X-100 have a positive influence for 95% of confidence with $R^2 = 89.0\%$.

A response surface methodology was done for pH 8.0–11.0, and the Triton X-100 concentration was 0.2–1.0%. The Pareto chart in **Figure 5** indicates that only pH and pH² have a positive influence.

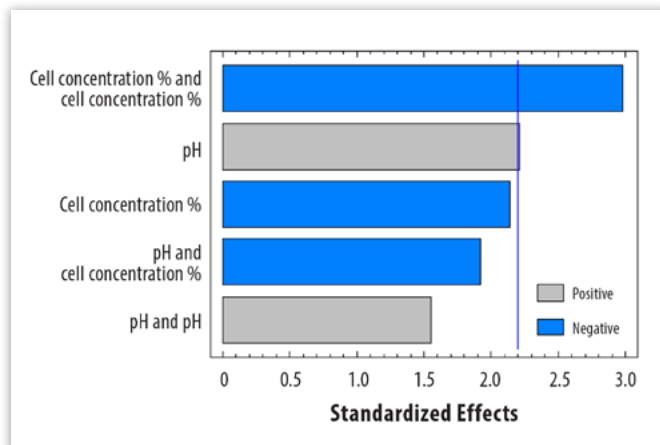


FIGURE 3. Pareto chart for pH and cell concentration on CIGB 550-E7 release yield.

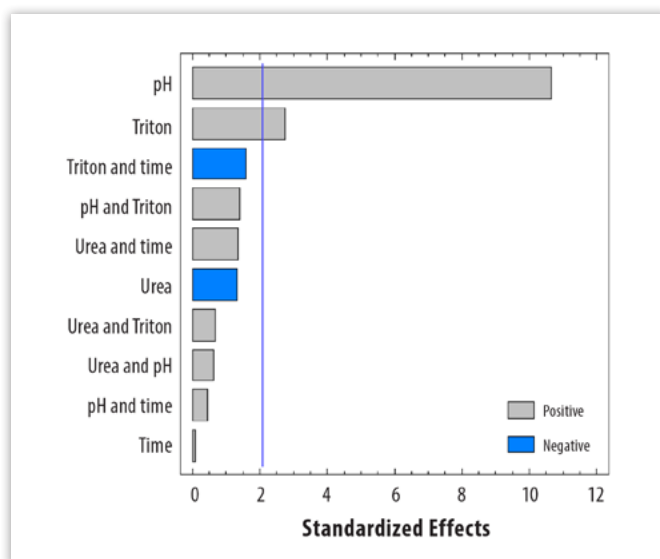


FIGURE 4. Pareto chart for the screening experiment design which shows the influence of independent variables on the chemical disruption and release of the target protein.

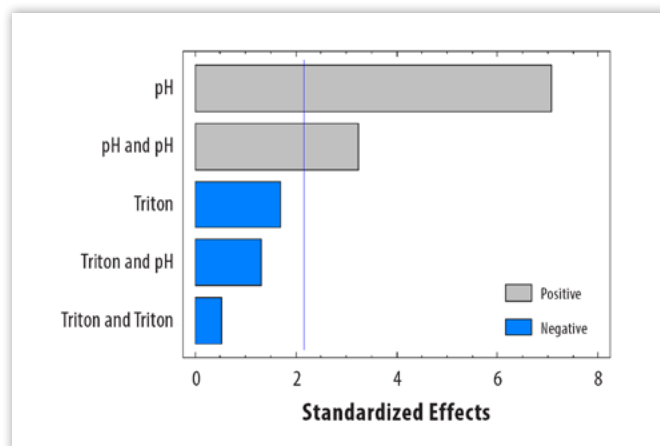


FIGURE 5. Pareto chart for response surface methodology on chemical cell disruption.

TABLE 5. Experimental results to confirm optimum yields using mechanical cell disruption.				
Run	Concentration (mg/mL)	Purity (%)	Yield (mg) CIGB 550-E7/g biomass	Relative Error (%)
1	4.641	25	11.810	2
2	4.899	24	11.660	4
3	4.591	25	11.490	5

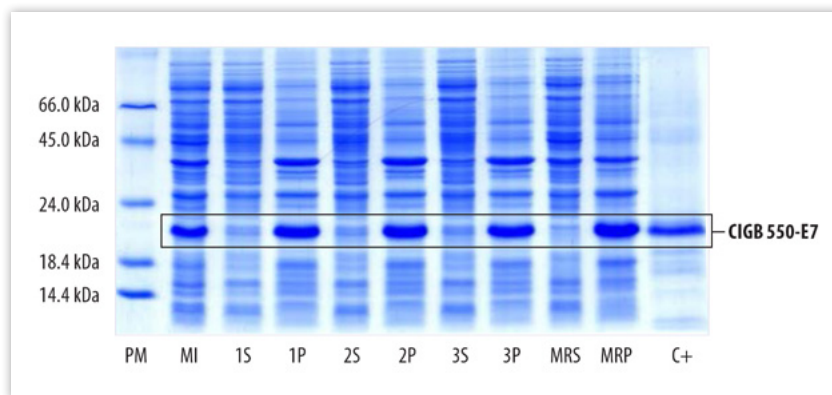


FIGURE 6. Electrophoresis under reducing conditions applying chemical and mechanical rupture. **PM:** molecular weight pattern; **MI:** initial sample; **1S–3S:** chemical rupture supernatant; **1P–3P:** chemical rupture precipitates; **MRS:** mechanical rupture supernatant; and **MRP:** mechanical rupture precipitates.

In order to optimize the process conditions for chemical cell disruption of *E. coli* cells for maximum possible release of CIGB 550-E7, multiple response optimization was done. The optimization predicted 1.18 mg CIGB 550-E7/mL using Triton X-100 at 1% and pH 10.2. The results were validated by conducting the experiments in triplicate, and the value obtained, 1.10 ± 0.02 mg CIGB 550-E7/mL, closely agrees with the value predicted.

Comparison Between Mechanical and Chemical Cell Disruption

SDS-PAGE analysis (**Figure 6**) shows compositions for soluble and insoluble fractions with mechanical and chemical methods. In both experiments, the CIGB 550-E7 protein is located in the insoluble fraction.

As described previously, two of the problems inherent in mechanical cell disruption are the release of nucleic acids and extensive cell fragmentation, both of which impact downstream steps. However, to select the method for the release of the target protein depends upon the target protein yield and the overall cost. Therefore, the purification of the CIGB 550-E7 protein was achieved (as described earlier) using mechanical and chemical cell disruption. The results show that the final fractions which contain the protein CIGB 550-E7 have similar purity in both cases: between 95–97%. Mechanical disruption yield and recovery were 1.24 and 1.37 times higher than values for the chemical disruption method (**Table 6**). Finally, the fraction

that contains the API CIGB 550-E7 had a yield of 6.14 mg of CIGB 550-E7/g of purified biomass. Moreover, the lipopolysaccharide contaminants were quantified in the final samples with an average of 31.25 EU/mL. This result fulfills the value established by the regulatory authority for an injectable.^[10] Previously, Granadillo *et al.* had reported^[8] 94% purity and 5.1 mg of purified CIGB 550-E7 per gram of biomass. However, contaminants such as lipopolysaccharides were higher than 10,000 EU/mL.

Biological Activity

To confirm the anti-tumor capacity of the proteins obtained for both cell disruption methods, a biological activity assay was conducted. C57BL/6 mice were immunized twice at one-week intervals with both proteins containing an alum adjuvant. Fourteen days after the last immunization, the mice were challenged with 5×10^4 TC-1 cells in the right leg. **Figure 7** shows that TC-1 cell implantation resulted in the appearance of palpable

TABLE 6. Comparative results for yield and recovery of chemical and mechanical cell disruption.

Samples Treated By	Yield (mg) CIGB 550-E7/g biomass	Recovery (%)
Chemical rupture	9.30 ± 0.10	54.00 ± 1.00
Mechanical rupture	11.60 ± 0.16	74.00 ± 1.00

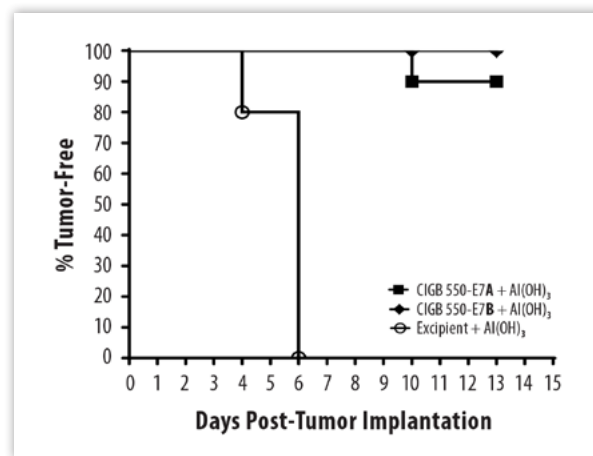


FIGURE 7. Evaluation of tumor response (tumor incidence) generated by immunization. CIGB 550-E7A: mechanical method; and CIGB 550-E7B chemical method.

subcutaneous tumors in all mice (10/10) immunized with the placebo between four and six days. In contrast, only 10% (1/10) of the mice vaccinated with protein CIGB 550-E7A, as measured by mechanical method, and 0% (0/10) of mice vaccinated with protein CIGB 550-E7B, as measured by chemical method, showed incidence of tumors at day 13. Compared with placebo administration, CIGB 550-E7A + alum and CIGB 550-E7B + alum immunizations show statistically significant protection against TC-1 tumor challenge ($p < 0.0001$, log-rank test) and no statistically significant differences between them ($p = 0.3173$, log-rank test). Therefore, with both methods of cell disruption, products with the same anti-tumor activity were obtained.

Cost/Benefit Analysis

A cost/benefit relationship (CBR) analysis was done by comparing each rupture method and the conventional process, which is the initial process with mechanical disruption without any optimization. A batch to produce 30g of biomass was established as a basis for calculation. CBR is evaluated using Equation 1.

$$CBR = \frac{Income^{NP} - Income^{CP}}{(OC^{NP} + IC^{NP} - OC^{CP})} \quad \text{(Equation 1)}$$

Where:

$Income^{NP}$: New process income

$Income^{CP}$: Conventional process income

OC^{NP} : New process operating cost

IC^{NP} : New process investment cost

OC^{CP} : Conventional process operating cost

Proportional costs for each rupture process and the conventional process are shown in **Table 7**. A negative CBR value for the chemical disruption process demonstrated that this alternative is not feasible.

TABLE 7. Comparison between each rupture method for the conventional process.

Analysis Parameters	Conventional Process	
	Chemical Disruption	Mechanical Disruption
Income	1.01	1.260
Operating cost	0.91	1.000
CBR value	-4.00	2.439

Conclusions

Attempts to maximize product recovery through increases in the intensity of cell disruption usually result in micronization of cell debris and increased viscosity of the product stream that is caused by the release of DNA and the co-release of contaminants. The results have shown that the final fractions containing the protein CIGB 550-E7 have similar purity for mechanical and chemical disruption, between 95–97%. In addition, the yield and recovery for mechanical disruption were 1.24 and 1.37 times higher than

values for chemical disruption methods. Each of the proteins obtained from both cell disruption methods showed a similar biological activity, so either method can be used. However, our results demonstrated that the mechanical method has greater economical advantage as compared to the chemical method. Finally, the process we developed produces an API that fulfills the requirements stipulated by the regulatory authorities in terms of purity, lipopolysaccharide contaminants, and biological activity.

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