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ORIGINAL RESEARCH

Optimization of Recombinant Human Interferon-Gamma Expression and Purification in *E. coli*, and Developing an Immunization Protocol for the Production of Optimal Humoral Response

Vanessa Uwituze[1,2], Claire M Mugasa[2], Monica Namayanja[2], David Paul Nalumenya[2], Jerome Ndayisenga[3], Kenneth Ssekatawa[4], Charles Drago Kato[2]

[1]Department of Medical Laboratory Science, Mount Kigali University, Kigali, Rwanda; [2]Department of Biotechnical and Diagnostic Sciences, Makerere University, Kampala, Uganda; [3]Research, Innovation, and Data Science Division, Rwanda Biomedical Center, Kigali, Rwanda; [4]Department of Science, Technical, and Vocational Education, Makerere University, Kampala, Uganda

Correspondence: Charles Drago Kato, Department of Biotechnical and Diagnostic Sciences, Makerere University, P.O Box 7062, Kampala, Uganda, Tel +256-703320705, Email katodrago@gmail.com

Background: Recombinant human interferon gamma (rhIFN-γ) and monoclonal antibodies are promising biomolecules for improving clinical medicines worldwide due to their biomedical applications. However, most African countries lack established production protocols. Although Freund's Complete Adjuvant (FCA) has been widely used to enhance antibody production, it has been reported to cause adverse effects. This study aimed to optimize the expression and purification of rhIFNγ and develop an immunization protocol for producing optimal humoral responses using alternative adjuvants such as RIBI Immunochem (RIBI) adjuvant and Freund's Incomplete Adjuvant (FIA), with FCA as the standard.

Methodology: The human interferon gamma (NM000619.3) sequence was retrieved from NCBI and sent to GenScript for codon optimization and construct synthesis. The constructs were transformed into *E. coli* BL-21, and protein expression was induced with IPTG. The expression and purification conditions were optimized. To evaluate immunization regimens and alternative adjuvants to FCA, three groups of female BALB/c mice were immunized with varying concentrations of rhIFNγ (25, 50, and 100 µg) mixed with an equal volume of RIBI adjuvant, FIA, and FCA. An Enzyme-Linked Immunosorbent Assay was used to measure antibody levels from each group.

Results: The study identified 0.6 mM of IPTG at 37°C and 250 rpm for 4 hours as the optimal conditions for rhIFNγ expression. Increasing imidazole concentration in wash and elution buffers enhanced rhIFNγ purity but reduced protein yield. Administration of 100 µg of rhIFNγ with RIBI adjuvant elicited a sufficient immune response, while higher concentrations of FCA and FIA increased side effects, indicating the need for lower adjuvant doses.

Conclusion: Although FCA has been reported to produce high antibody titers with notable side effects, this study found no significant difference in antibody levels between mice immunized with 100 µg rhIFNγ mixed with FCA and the group immunized with 100 µg rhIFNγ plus RIBI adjuvant.

Keywords: recombinant human interferon-gamma, RIBI immunochem adjuvant, Freund's incomplete adjuvant, humoral response

Introduction

Recombinant human interferon-gamma (rhIFNγ) and its monoclonal antibodies are among the most lucrative biopharmaceuticals, with an estimated market increase of $ 8 billion by 2024 and projected to be $ 12 billion by 2030.[1] The increase in market size is due to their utilization in clinical medicine, particularly in diagnosis, research, and vaccine development. Indeed, anti-viral, anti-bacterial, and anti-cancer activities of rhIFNγ increase their therapeutic efficacy against a wide range of diseases.[2] Despite the applications of these biomolecules, most African countries lack a production pipeline.[3] Africa's population contributes significantly to the global population; the continent is projected to be home to 27% of the global population by 2050.[4] With the

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continued growth of the global population, the world remains off track in achieving global health sustainability, particularly in securing equitable access to vaccines and therapeutics needed to respond effectively to pandemics and emerging and re-emerging diseases.[5] The African continent relies on other continents for the production and supply of drugs, vaccines, recombinant diagnostics, and therapeutics, and this has created a significant health risk on the continent.[6] It is estimated that about three million children under five years of age die each year in Africa due to preventable diseases.[7] Recent strategies for disease management and prevention require the participation of the entire world. Africa contributes approximately 18% of the global population, 25% of the global disease burden, and 50% of infectious diseases originate in Africa; however, less than 4% of diagnostics, therapeutics, and vaccines used on the continent are produced in Africa.[8] Most of the vaccines of the past decades were live attenuated or inactivated pathogens and faced some issues, including but not limited to the possibility of the weakened organisms mutating and regaining their pathogenicity, and also elicited a weaker immune response.[9] The recent advancement in synthetic biology and molecular engineering has opened possibilities for designing and developing recombinant protein-based vaccines, such as subunit recombinant vaccines. The protein-based vaccines, therapeutics, and diagnostics have evolved from hosts such as *E. coli, Saccharomyces cerevisiae*, insects, and mammalian cells, and they are promising to revolutionize the safety and increase therapeutic vaccine acceptance across the globe.[10]

Escherichia coli (E. coli) remains the most commonly used expression system for the production of heterologous proteins because of its simple nutrient requirements, high growth rate, well-understood physiology, and molecular genetics.[11] The use of *E. coli* for investigating the expression of rhIFNγ faces a significant challenge due to the formation of insoluble intracellular inclusion bodies, where the proteins are partially or entirely denatured, leading to protein unfolding and unglycosylation.[12] However, in recent years, various techniques have been developed to produce active recombinant proteins in *E. coli*, including dilution, combined urea-ammonium detergent, ammonium detergent-assisted, and chromatography refolding.[13] A good recombinant protein must have a long shelf life, high efficiency, and safety, which could be achieved through optimization.[14] This ensures the stability and reliability of recombinant protein expression in *E. coli*, thereby enabling subsequent immunological investigations.[15] Previous methods that have been developed for the purification of rhIFNγ include CM-Sepharose, size exclusion chromatography, and cation exchange chromatography, which are complex and have shortcomings.[16] They require specialized columns, which increase the production cost; hence, most low- and middle-income countries (LMICs) can hardly afford their adoption. In this study, we developed a method that could allow the production of rhIFNγ in a simple, affordable, and efficient manner.

The production of rhIFNγ is currently limited to one USA commercial company, despite the worldwide need in the treatment of granulomatous diseases, severe malignant osteopetrosis, hepatitis B, and multidrug resistance in Mycobacterium tuberculosis.[17] Recombinant proteins, such as rhIFNγ, are potentially used to elicit an immune response in immunised animals during antibody production, which can be further enhanced with adjuvants.[18] The use of adjuvants in animal research requires careful consideration, as they can cause severe adverse effects, such as pain, swelling, and distress in the research animals, particularly laboratory mice.[19] Highly potent inflammatory agents, particularly Freund's Complete Adjuvant (FCA), cause severe effects such as inflammation, granulomas, abscesses, and even systemic issues in research animals.[20] The laboratory animal welfare guidelines issued by Institutional Animal Care and Use Committees (IACUCs) recommend using alternative, less problematic adjuvants to laboratory animals, such as the Research Immunology Biological Institute (RIBI) adjuvant system and Freund's incomplete adjuvant (FIA).[21] Despite these side effects, researchers continue to prefer FCA because the adjuvant has proven to stimulate a higher humoral response than most alternative methods.[22] Therefore, this study aimed to develop a simple, cost-effective, and efficient method for the production of recombinant human interferon-gamma suitable for resource-limited settings. In addition, the study evaluated alternative adjuvants, including the Research Immunology Biological Institute (RIBI) adjuvant system and Freund's incomplete adjuvant, in comparison with Freund's complete adjuvant, to optimize antibody production while minimizing adverse effects in laboratory animals.

Materials and Methods

Study Design

This was a laboratory-based experimental study aimed at optimizing the expression and purification conditions of rhIFNγ in *E. coli* and developing an immunization protocol for use in the production of an optimal humoral response against it.

This study also evaluated the efficacy of alternative adjuvants to Freund's Complete Adjuvant (FCA) for eliciting an optimal humoral immune response in the production of monoclonal antibodies. The experimental work was conducted at the Center for Biosecurity and Global Health, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University.

Materials

Escherichia coli BL-21(DE3) was obtained from Novagen (SAF-69450-4). RIBI adjuvant (S6322), anti-His antibodies (SAB5600096), and Interferon-γ human (I3265) were procured from Sigma-Aldrich. IPTH (GC6586-5G) was obtained from Glentham Life Science, while the protein ladder (26619), Freund's Complete Adjuvant (FCA; 77140), and Freund's Incomplete Adjuvant (FIA; 77145) were sourced from Thermo Fisher Scientific. Anti-human interferon-gamma antibodies (MBS670031) were obtained from MyBiosource USA.

Preparation of pET-28a (+)-hIFNγ Constructs and Transformation into E. coli BL-21 (DEC3)

The coding sequence of human interferon-gamma (Interferon gamma) was retrieved from the National Center for Biotechnology Information database (accession number NM000619). The sequence was optimized for expression in *Escherichia coli* BL21 (DE3) using the proprietary codon-optimization algorithm provided by GenScript. Codon optimization was performed to improve translational efficiency in *E. coli* by adapting the human gene sequence to the codon usage bias of the bacterial host. The optimization process involved: (i) replacing rare codons with synonymous codons preferred by *E. coli*, (ii) increasing the codon adaptation index (CAI) toward values close to 1.0, (iii) adjusting GC content to match the typical range for efficient expression in *E. coli* (30–70%), (iv) removing potential mRNA secondary structures that could interfere with translation initiation, and (v) eliminating internal restriction enzyme recognition sites that could interfere with cloning. The optimized gene was synthesized and cloned into the expression vector between the restriction sites HindIII (AAGCTT) and XhoI (CTCGAG). Lyophilized human interferon-gamma gene constructs in the pET-28a (+) vector (plasmid) were received from GenScript and centrifuged at 10,000 rpm for 2 min. Thereafter, 20 µL of nuclease-free water was carefully added to the tube to reconstitute the constructs as directed by GenScript. The pET-28a (+)-hIFNγ constructs were transformed into *E. coli* BL-21 cells using the heat shock method as outlined in the New England Biolabs transformation protocol (C2528).[23] Thereafter, plasmid DNA was extracted from selected transformed colonies using the Qiagen Plasmid Mini Kit according to the manufacturer's instructions to confirm the presence of the insert.[24] The isolated plasmids were used as templates for PCR amplification of the recombinant human interferon-gamma (rhIFNγ) gene using T7 promoter and terminator primers. PCR conditions were optimized as follows: initial denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 1 minute, annealing at 45°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Successful transformation and amplification were confirmed by the presence of a 510 bp PCR product corresponding to the rhIFNγ gene.

Selection of the Best Expressing Colony

Four successfully transformed colonies were selected from the LB agar plate, and each was inoculated into 10 mL of fresh LB broth containing 50 µg/mL kanamycin. The cultures were incubated overnight at 37°C with shaking at 180 rpm. The following day, each overnight culture was subcultured under the same conditions and incubated at 37°C, 250 rpm until the optical density at 600 nm (OD_{600}) reached approximately 0.6. At this point, 1 mL of each culture (pre-induced or non-induced sample) was archived at –80°C. The remaining cultures were induced with 1 mM IPTG and incubated further at 37°C, while shaking at 250 rpm. Induced and non-induced samples were prepared for SDS-PAGE by resuspending cell pellets in 50 µL of SDS loading buffer (deionized water, 0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, and 10% APS), followed by heating at 100°C for 5 minutes. Protein expression was analyzed on a 15% SDS-PAGE gel, and band intensity was quantified using ImageJ software to identify the highest-expressing colony. Western blot analysis using anti-human interferon-gamma antibodies confirmed successful expression of the recombinant human interferon-gamma (rhIFNγ) protein. Interferon-γ human (I3265) obtained from Sigma was used as the positive control.

Optimal Time Conditions for Expression (Time, IPTG, and Temperature)

To determine the optimal time for recombinant human interferon-gamma (rhIFN-γ) expression, fresh bacterial colonies were cultured, and protein expression was induced as previously described. Following induction, cultures were incubated for 7 hours, and samples were collected at 1-hour intervals. Each collected sample was centrifuged at 12,000 rpm, and the resulting pellets were resuspended in SDS loading buffer before being resolved on a 15% SDS-PAGE gel. The optimal temperature for protein expression was evaluated by inducing expression at 37°C and 20°C under identical conditions. To determine the optimal IPTG concentration, cultures were prepared as described above and induced with varying IPTG concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.8, and 2.0 mM). Induced cultures were incubated at 37°C with shaking at 250 rpm for 4 hours. After incubation, samples from each IPTG concentration were collected and analysed by SDS-PAGE. Protein band intensity was quantified using ImageJ software to determine relative expression levels.

Recombinant Human Interferon-Gamma Fraction Expressed

Fresh bacterial culture (50 µL) was inoculated into 10 mL LB medium containing 50 µg/mL kanamycin and incubated overnight at 37°C. The overnight culture (8 mL) was then subcultured into 400 mL LB with kanamycin and grown to an OD_{600} of 0.6. A pre-induced sample was collected, and protein expression was induced with 0.6 mM IPTG for 4 hours at 37°C with shaking. An induced sample was collected before harvesting the remaining cells by centrifugation. The cell pellet was resuspended in lysis buffer (50 mM NaCl, 50 mM Tris-Cl) with lysozyme and incubated, followed by treatment with deoxycholic acid. The lysate was homogenized, and samples were collected. After centrifugation, supernatant (S1) and pellet fractions were separated. The pellet was washed sequentially with PBS containing 0.1% Triton X-100 and distilled water, with centrifugation between washes.

The washed pellet (inclusion bodies) was resuspended in solubilizing buffer (50 mM NaCl, 50 mM Na_2HPO_4, 10 mM imidazole, 8 M urea). Inclusion body (IB), solubilized inclusion body (SIB), and clear inclusion body (CIB) samples were collected after incubation and centrifugation. All collected samples were mixed with SDS loading buffer and analyzed by SDS-PAGE.

Batch Purification Under Native Conditions

One milliliter of the 50% Ni-NTA slurry was added to 4 mL of cleared lysate and mixed gently by shaking at 200 rpm on a rotary shaker at 4°C for 60 minutes. The lysate -Ni-NTA mixture was loaded into a column with a capped bottom outlet as described in Protein purification. The bottom cap was removed, and the flow-through was collected for SDS analysis. The column was washed twice with 4 mL of wash buffer (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM Imidazole, pH 8.0), and washes were collected for SDS-PAGE analysis. The protein was eluted with 0.5 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.0), and eluates were collected for SDS-PAGE analysis.

Optimizing Imidazole in Wash Buffer

To determine the optimal imidazole concentration for high protein purification and yield in the wash buffer, 4 mL of the 50% Ni-NTA slurry was added to 30 mL of cleared lysate and mixed gently by shaking at 200 rpm and on a rotary shaker at 4°C for 60 minutes. Two millilitres of the lysate-Ni-NTA mixture were loaded into 5 different columns with a bottom outlet capped. The bottom cap was removed, and the flow-through was collected. The 5 washing buffers were prepared, each containing a specific concentration of imidazole (10 mM, 20 mM, 40 mM, 50 mM, 60 mM). Each column containing lysate was washed with a buffer containing a specific concentration of imidazole. The wash fractions from each column were collected for SDS-PAGE analysis. The results showed that the increase in imidazole concentration in the wash buffer improves the purity of the protein but reduces the yield of the protein.

Optimizing Imidazole in the Elution Buffer

The study also explored the effects of adding imidazole to the elution buffer for purifying proteins. Eight millilitres of the 50% Ni-NTA slurry were added to 60 mL of cleared lysate and mixed gently by shaking at 200 rpm and on a rotary shaker at 4°C for 60 minutes. Two millilitres of the lysate-Ni-NTA mixture were loaded into 7 different columns with a bottom outlet capped. The bottom cap was removed, and the flow-through was collected for SDS-PAGE analysis. Each column was then washed 4 times with a washing buffer containing (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM Imidazole, and 8 M urea, pH 8.0). Protein from each column was then eluted with an elution buffer containing a specific concentration of imidazole 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM), each with (500 mM NaCl, 50 mM NaH2PO4,8M urea, pH 8.0). Eluates from each column were collected for SDS PAGE analysis.

Quantification of the Purified Protein

This experiment was done to determine the concentration of recombinant human interferon gamma using Qubit protein Assay kits. The Qubit working solution was prepared by diluting the Qubit protein reagent by 1:200 in the Qubit protein buffer. The mixture was incubated at room temperature for 5 minutes; thereafter, 1 µL was discarded from 200 µL and replaced with 1 µL of the protein sample, followed by an additional 5 minutes of room temperature incubation. Thereafter, the protein concentration was measured using a Qubit Fluorometer, following the manufacturer's instructions and guidelines.[25]

Protein Dialysis

Dialysis was performed using a semi-permeable regenerated cellulose membrane to remove salts and urea from our protein before the animal study. The cellulose membrane was regenerated using a regeneration buffer containing 2% sodium bicarbonate and 1 mM EDTA. Recombinant human interferon gamma (rhIFNγ) was loaded into the dialysis tubing and immersed in dialysis buffer containing 1× PBS, then placed on a magnetic stirrer and stirred at 39 rpm for 2 hours at room temperature. The dialysis buffer was replaced twice at two-hour intervals. The dialyzed protein was then collected and analysed using 15% SDS-PAGE. Anti-His Western blotting was subsequently performed to confirm the presence of rhIFNγ.

Determining the Best Immunizing Regimen

The study utilized a total of 39 female BALB/c mice, randomly selected and obtained from CeBIGH. The mice were approximately 6 weeks old, with an average weight of 9 ± 1 g, and were housed in standard cages for two weeks of acclimatization under controlled laboratory conditions (temperature: 26 ± 3°C, relative humidity: 54 ± 5%, and normal light cycles). The sample size was determined using GitHub software. This experiment was conducted to determine the optimal dose and immunization regimen, and to evaluate the efficacy of alternative adjuvants to Freund's Complete Adjuvant (FCA) in inducing sufficient antibody responses. For each experiment, four groups of three mice each were established:

Group 1: 25 µg rhIFNγ + FCA
Group 2: 50 µg rhIFNγ + FCA
Group 3: 100 µg rhIFNγ + FCA
Group 4 (control): FCA + PBS

The same design was repeated using RIBI adjuvant and Freund's Incomplete Adjuvant (FIA). Additionally, a PBS-only control group (three mice) was included for all experiments. In the FCA and RIBI groups, mice were boosted with the same antigen dose every three weeks, while in the FIA group, boosting occurred after two weeks, due to its reported lower immune stimulation. Serum samples were collected before each booster, and antibody titers were determined using an in-house ELISA. The mouse exhibiting the highest antibody titer received a final booster with antigen alone, and 3 days later, it was euthanized for spleen harvesting for hybridoma development, which will be reported separately. After the experimentation period, all the mice were euthanized by administering 100% carbon dioxide (CO_2) as earlier outlined by Turner et al.[26]

Assessment of Immune Response Using ELISA

The antibody response generated against the purified recombinant human interferon-gamma was assessed using an indirect ELISA. Microplate wells were coated with 100 µL of carbonate-bicarbonate buffer containing 1 µg/mL of the recombinant antigen and incubated overnight at 4°C. After incubation, the unbound protein was discarded, and the plates

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were washed once with 200 µL of PBS containing 0.5% Tween-20 (PBST). The wash buffer was allowed to stand for five minutes at room temperature with gentle agitation before being removed, and two additional washes were performed. Pre-immune and immune sera were diluted 1:1000 in PBS. For each animal, 150 µL of serum was added to the wells in the first row following a predetermined layout, while 100 µL of PBS was added to the remaining wells. Serial two-fold dilutions were prepared down the plate using a multichannel pipette by transferring 50 µL from one row to the next, mixing between transfers. Plates were then sealed and incubated for two hours at room temperature. Following incubation, wells were emptied and washed five times with PBST. Subsequently, 100 µL of rabbit anti-mouse IgG secondary antibody (Sigma-Aldrich), diluted 1:10,000 in blocking buffer, was added to each well. The plates were then incubated for 2 hours at room temperature. After incubation, the wells were washed five times with PBST to remove unbound antibody; thereafter, 100 µL of TMB substrate was added to each well and allowed to develop in the dark for 15 minutes. The enzymatic reaction was stopped by adding 100 µL of 2 M sulfuric acid, and absorbance was recorded at 450 nm using an ELISA reader. Antibody titers were calculated by plotting optical density against serum dilution for both immune and pre-immune samples. The titer was taken as the dilution immediately preceding the point at which the immune and pre-immune curves converged, indicating the limit of detectable specific antibody.

Data Presentation and Analysis

Descriptive statistics data presentation was conducted to express outcomes on data from optimization of the conditions for expression and purification of recombinant human interferon gamma. Data analysis was performed using GraphPad version 7 statistical software. The data were normally distributed. Two-way ANOVA was used to determine the difference in antibody titres within the same group, and Student's test was used to determine the difference in antibody titres within the same group. Values for each group were expressed as mean ± SEM at a significant level $\alpha = 0.05$.

Results

Successful Production of the Expression Construct

To improve expression levels, the sequence NM000619.3 was codon optimized by Genscript. Codon optimization was reported to improve protein expression levels by increasing translation efficiency. Codon optimization is a method that is commonly used to increase the expression of biotherapeutic recombinant proteins through the use of synonymous codon mutations in messenger RNA (mRNA) coding regions, achieved by changing the nucleotide sequence without changing the sequence of amino acids. Codon optimization of the human interferon-gamma gene resulted in an increase in the predicted codon adaptation index (CAI) from approximately ~0.63 (native sequence) to ~0.92, indicating improved compatibility with the codon usage bias of *E. coli*. Furthermore, the codon optimization increased the GC content from 39 to 44.71%. Also, rare codons that could potentially limit translation efficiency were replaced with synonymous codons frequently used in *E. coli*. No unwanted restriction enzyme recognition sites or premature transcription termination signals were detected in the optimized sequence.

Selecting the Best-Expressing Colony for Expression and Purification Optimization

To determine the best colony to be used for downstream activities of expression and purification of recombinant human interferon-gamma. Four colonies named 1,2,3,4 were selected for mini-expression. SDS-PAGE results showed that all 4 selected colonies had good expression, evidenced by thick bands of the expected size of rhIFNγ of 17 kDa (Figure 1A). Western blotting using anti-His antibodies confirmed that the expressed protein was the rhIFNγ recombinant human interferon-gamma protein (Figure 1B). The integrated optical density of protein per lane was quantified using Image J. Colony 4 had the highest relative density, followed by 1, then 2, and then 3, respectively (Figure 1C). Hence, colony four was used for the downstream activities of optimization of expression and purification conditions.

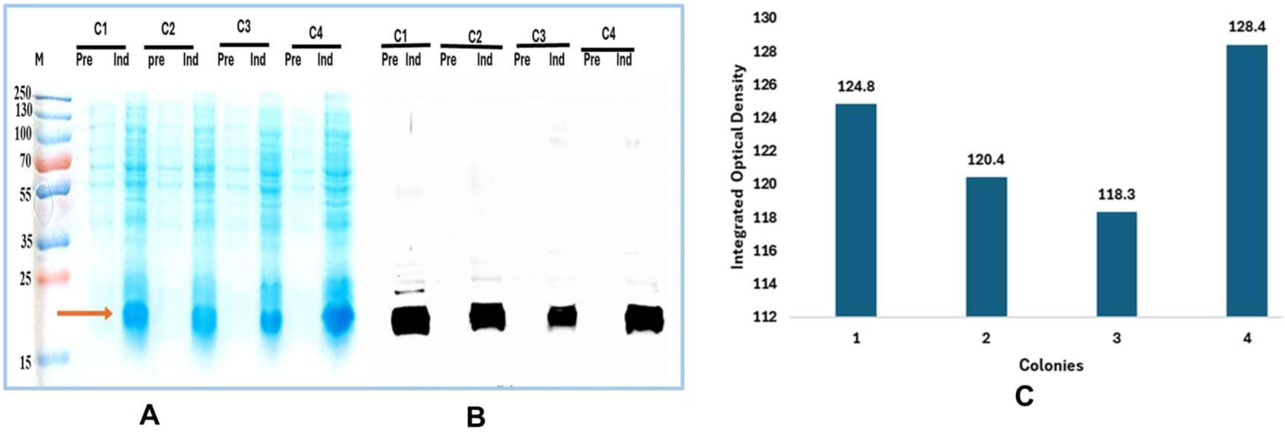

Figure 1 SDS-PAGE electrophoresis for the determination of the best-expressing colony. Non-induced samples and induced rhIFNγ protein from selected colonies were loaded on electrophoresed 15% SDS-PAGE to determine the best-expressing colony, anti-His Western blotting was performed to confirm successful expression of rhIFNγ. Where (**A**) is a 15% SDS–PAGE gel showing the expression results of 17kDa rhIFNγ, (**B**) is the corresponding Western blot probed with mouse anti-His IgG and (**C**) is the integrated optical density of protein per lane calculated using Image J. In (**A**) M is PageRuler™ plus Prestained Protein Ladder, and in both A and B, C is colony (where C1-C4 are the respective colonies), Pre is pre–induced, and Ind is induced. The best-expressing colony is Four. The arrow indicates the bands corresponding to rhIFN-γ at ~17 kDa.

Optimal Conditions for Expression of Recombinant Human Interferon Gamma (Time, IPTG, and Temperature)

Recombinant human interferon gamma hIFNγ protein expression increased with time after IPTG induction; the highest amount of protein expression was obtained at four hours, and thereafter, it started to decline gradually. Thus, the optimal time for expression was revealed to be 4 hours from this study (Figure 2). To determine the optimal IPTG for expression of recombinant human interferon gamma, expression was induced using different concentrations of IPTG from 0.2 mM to 2 mM. The level of expression was estimated through Image J analysis by determining the integrated density of every lane. Findings revealed that the protein expression level was high and constant from 0.6 to 2 mM (Figure 3A and B). The 0.6 mM was concluded as the optimal IPTG concentration for inducing the expression of recombinant human interferon-gamma in *E. coli* BL21(DE3) cells from this study. To determine the best temperature for the expression of rhIFNγ, expression was done at 37°C and 250 rpm for 4 hours, and 20°C overnight at 250 rpm. Expression at 37°C and 250 rpm showed a higher yield of protein expression (Figure 4).

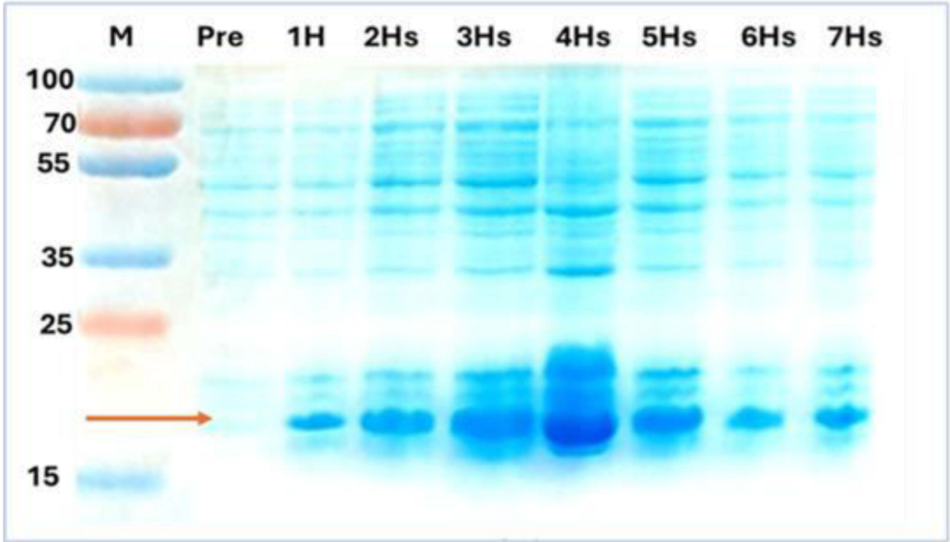

Figure 2 SDS-PAGE analysis to determine the optimal time for expression of rhIFNγ. A 15% SDS–PAGE showing the expression levels of rhIFNγ expression post-induction, M is the PageRuler™ Plus Prestained Protein Ladder, and 1H-7Hs is the hours post-induction. The arrow indicates the bands corresponding to rhIFN-γ at ~17 kDa.

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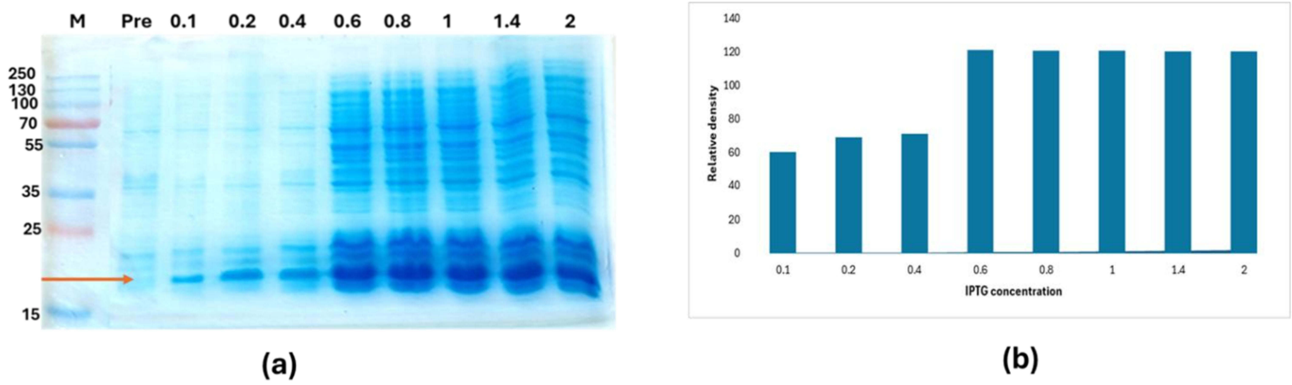

Figure 3 SDS-PAGE analysis of expression of rhIFNγ to determine the optimal IPTG concentration for expression of rhIFNγ (**a**). Where M is PageRuler™ Plus Prestained Protein Ladder, Pre is Uninduced, and 0.1 to 2 are different IPTG concentrations (mM). (**b**) The integrated Optical Density per lane was calculated using Image J. Zero point six is the optimal IPTG from this experiment. The arrow indicates the bands corresponding to rhIFN-γ at ~17 kDa.

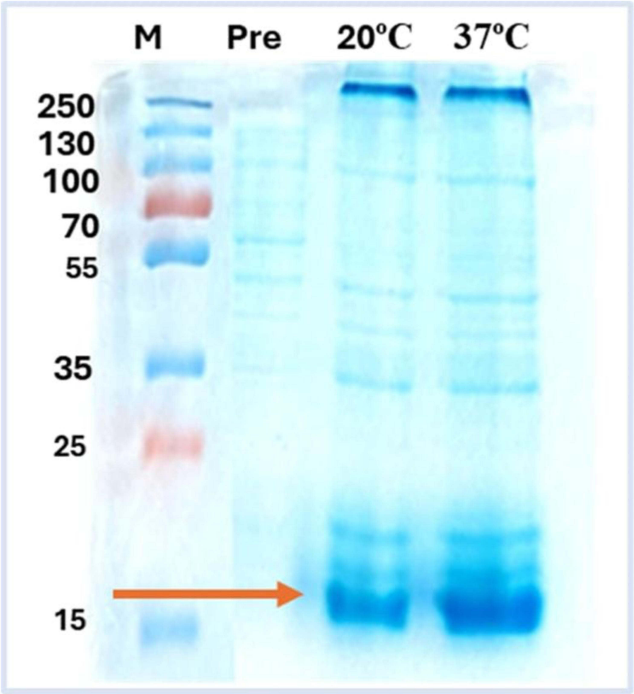

Figure 4 SDS-PAGE analysis of expression of rhIFNγ to determine the optimal temperature for expression of rhIFNγ. Where M is PageRuler™ Plus Prestained Protein Ladder, Pre is Uninduced. The arrow indicates the bands corresponding to rhIFN-γ at ~17 kDa.

Determining the Fraction That Contains the rhIFNγ Protein

Depending on the fraction in which protein is expressed, protein can either be purified under soluble or native conditions. To determine whether recombinant human interferon-gamma should be purified under native or denaturing conditions. Recombinant human interferon-gamma expression was in inclusion bodies (Figure 5), which suggests that purification is optimal under denaturing conditions.

Optimal Conditions for Purification of Recombinant Human Interferon-Gamma

To optimize the purification of recombinant human interferon-gamma, several purification protocols were evaluated. In the first protocol, purification was performed under denaturing conditions using 8 M urea to solubilize the inclusion bodies, followed by elution based on pH change. Although this approach produced a high protein yield of 2250 ± 164 µg/mL (mean ± SD), it failed to

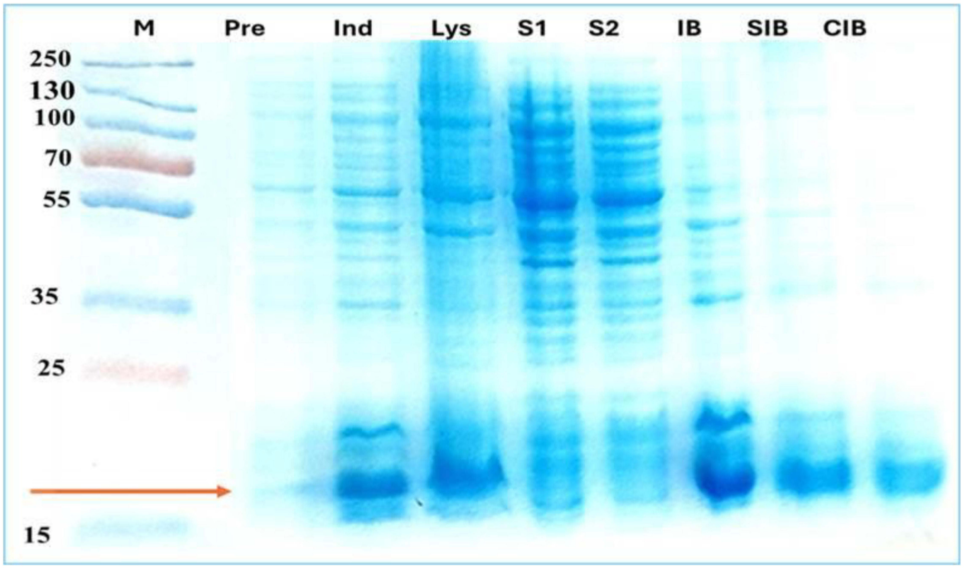

Figure 5 SDS-PAGE analysis of expression products to determine the fraction that contains rhIFNγ Protein. Fractions from lysed and solubilized rhIFNγ protein were loaded in electrophoresed 15% SDS-PAGE to determine the fraction that contains rhIFNγ. Where M is Page Plus Prestained Protein marker, Pre: pre-induced, Ind: induced, Ly: lysate, S1: is supernatant 1, S2: Is Supernatant 2, IB is Inclusion Bodies, SIB: Solubilised Inclusion bodies, CIB is Clear solubilized bodies. The proteins were in a fraction of inclusion bodies. The arrow indicates the bands corresponding to rhIFN-γ at ~17 kDa.

yield a pure protein preparation (Figure 6A). In the second protocol, 8 M urea was again used for denaturation, but imidazole was employed for both washing and elution. Different imidazole concentrations (10–60 mM) were tested in the wash buffer to minimize non-specific binding. Increasing the imidazole concentration in the wash buffer improved protein purity but reduced yield (1750 ± 149 µg/mL) (Figure 6B). Various imidazole concentrations in the elution buffer were also examined to identify conditions that produced the highest protein yield; however, no significant difference was observed in protein concentration across the range of 400 to 700 mM (Figure 6C). Following optimization, purification was carried out using 60 mM imidazole in the wash buffer and 400 mM imidazole in the elution buffer for large-scale protein expression. The purified protein remained stable after dialysis with a final yield of 1640 ± 123 µg/mL (Figure 7A and B).

Antibody Concentration Across the Groups

All mice increased weight from the average of 10g ± 1 to the average of 25g ± 1 and there was no death during the period of study. And no observable side effects were recorded in animals that received the RIBI adjuvant. The findings showed

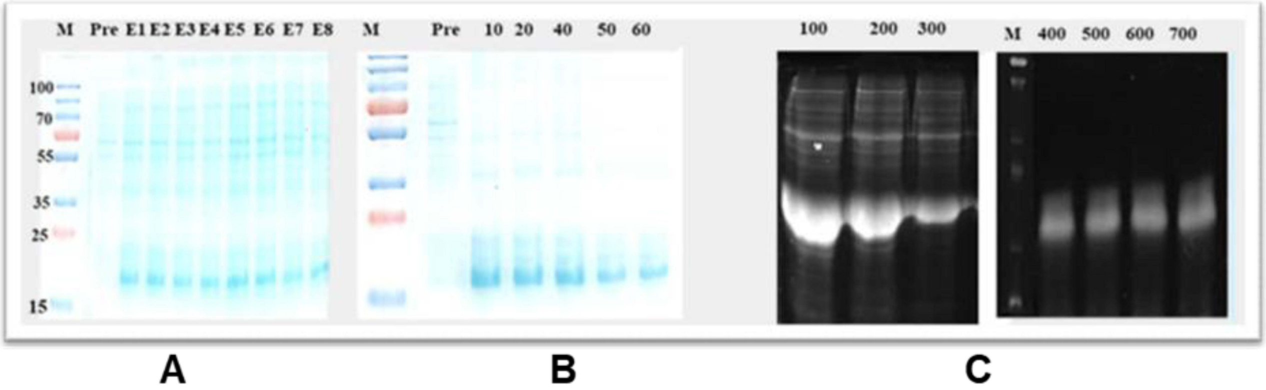

Figure 6 SDS-PAGE analysis of expression products from various protocols to determine the optimal protocol for purification. Elution and washes collected from different protocols were loaded on electrophoresed 15% SDS-PAGE to determine the optimal protocol for the purification of rhIFNγ. Where (**A**) Purification under denaturing conditions, using different pH to elute the protein, (**B**) Optimum concentration of imidazole in the wash buffer, and (**C**) Optimizing the concentration of imidazole in the elution buffers. In all, M is a protein Marker, and in (**A**) E stands for Elution.

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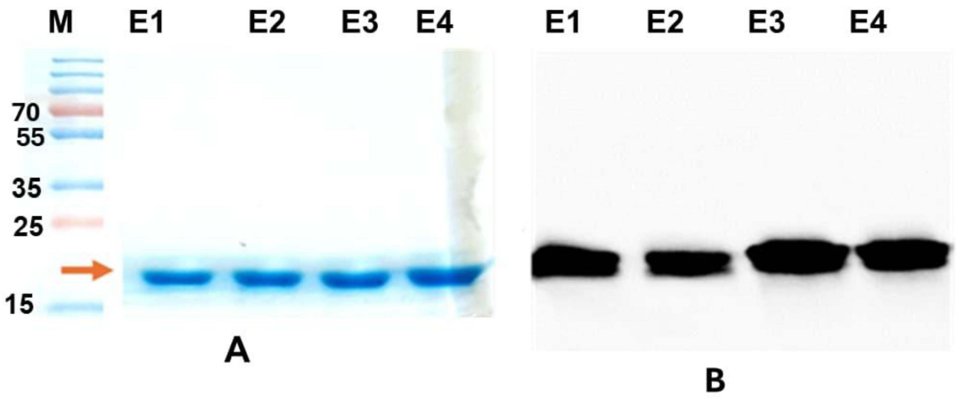

Figure 7 SDA-PAGE and Western Blotting analysis for the purity of the produced rhIFNγ. The elutions collected from purified rhIFNγ protein were electrophoresed on 15% SDS-PAGE. Where (**A**) is 15% SDS PAGE showing the pooled elution fractions and (**B**) The membrane probe with mouse anti-His and respective secondary antibody. The arrow indicates the bands corresponding to rhIFN-γ at ~17 kDa.

that each adjuvant tested elicited an immune response, while no such response was observed in the control groups (Figure 8a–d). However, the intensity of the response varied with the dose, as presented in Figure 9a–c). Statistical analysis revealed that several boosts and antigen concentration have a significant effect on the levels of antibodies (Figure 8) except in the controls. FCA had the highest relative antibody levels throughout the study (8a), followed by RIBI (8b), and then FIA (8c); except that after the third immunization (after the second boost), the group that received 25 µg of rhIFNγ with FIA had a higher relative antibody level than the group that received 25 µg of rhIFNγ and RIBI (Figure 9a). Two-way ANOVA analysis shows that at a (CI of 95, F(3, 16) = 0.9418, $p = 0.4436$, $\eta^2 = 2.039\%$), there was no significant difference in relative antibody level generated by those groups. Among the group that received FCA, 100 µg produced the highest antibody titer, followed by 50 µg, then 25 µg (Figure 8a). Boosting and dose (antigen

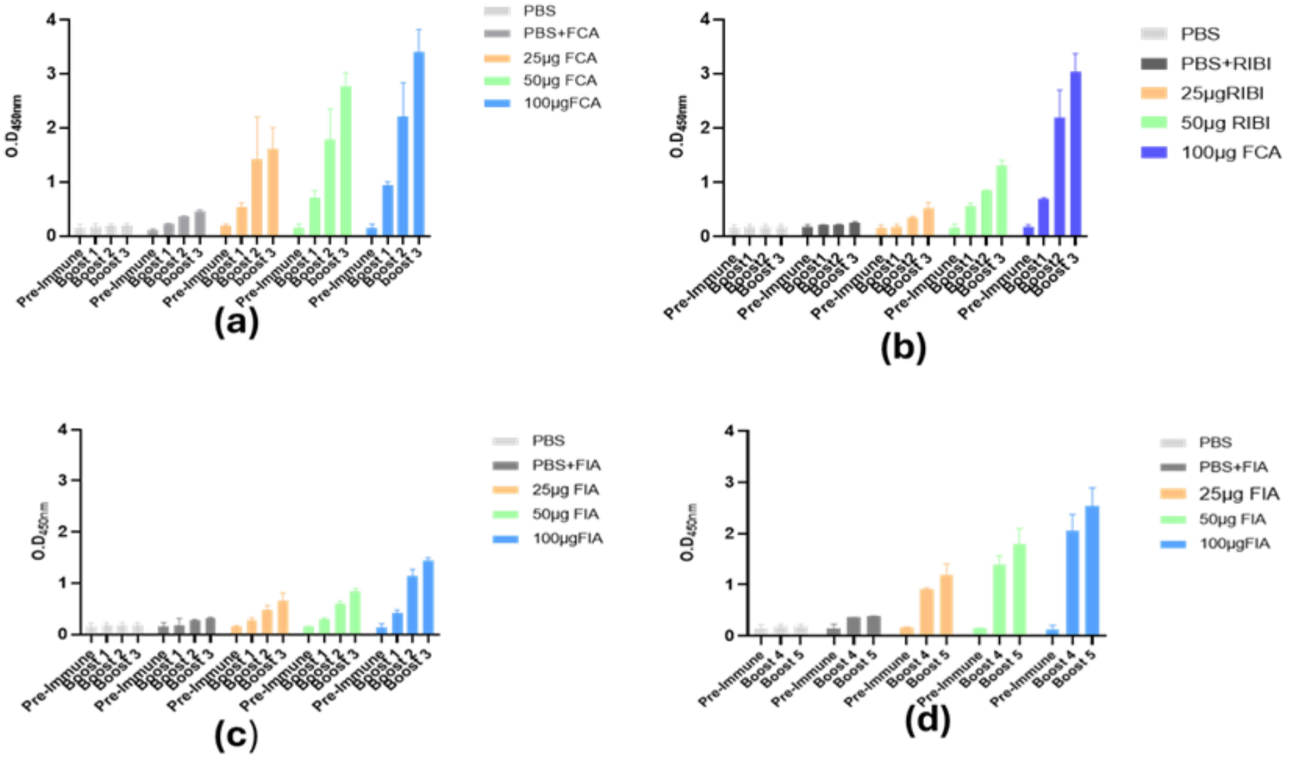

Figure 8 Antibody response generated by mice immunized with FCA, RIBI, and FIA, each mixed with purified rhIFNγ. Where (**a**) represents the antibody concentration generated by a group immunized with FCA, (**b**) A group immunized with RIBI adjuvant, and (**c**) and (**d**) are groups that were immunized with FIA adjuvant.

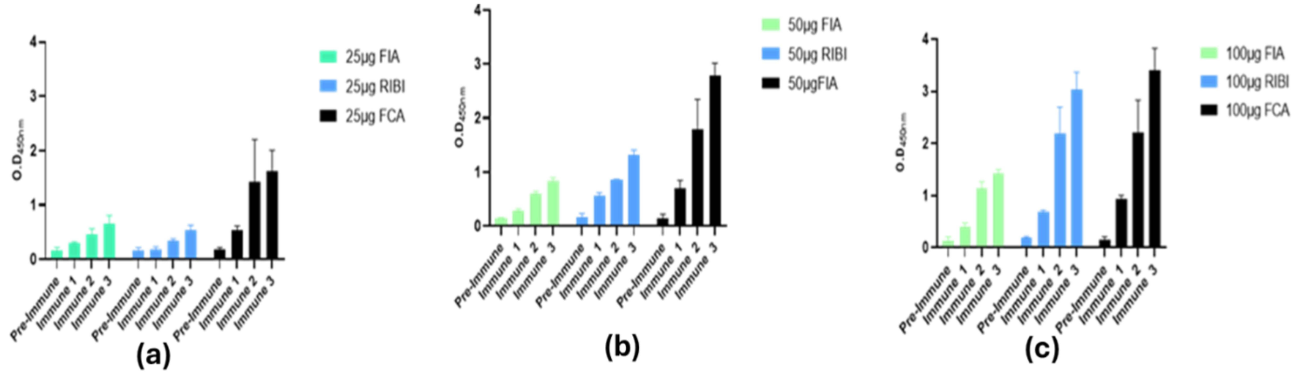

Figure 9 Antibody response generated by different concentrations of rhIFNγ mixed with adjuvants. Where (**a**) is 25 µg, (**b**) is 50 µg, and (**c**) is 100 µg across all adjuvants.

concentration) showed a significant effect on relative antibody level in this group at CI 95, $p > 0.001$ except that there was no significant difference between the relative antibody level generated by a group immunized with 50 µg rhIFNγ and FCA and those immunized with 100 µg of rhIFNγ and FCA after 3 immunizations at (CI 95, F(3, 16) = 0.8832, $p = 0.4707$, $\eta^2 = 0.9683\%$). Among the group that received rhIFNγ and RIBI adjuvant, at CI 95, F(12, 40) = 43.24, $p < 0.0001$, $\eta^2 = 31.40\%$, boosting and concentration of protein had a significant effect on the antibodies' reactivity. For this group, 100 µg of rhIFNγ and RIBI produced the highest antibody levels, followed by 50 µg, then 25 µg (Figure 8b). In the group that received rhIFNγ and FIA, 100 µg produced the highest relative antibody levels, followed by 50 µg, then 25 µg. The 4th and 5th boosting considerably increased the antibody levels in the rhIFNγ and FIA immunized group (Figure 8d). Among all adjuvants, FCA produced the highest antibody response, followed by RIBI, then FIA, and a dose of 100 µg produced the highest antibody reactivity, followed by 50 µg, then 100 µg, as presented in Figures 9a–c. And there was no significant difference between the relative antibody levels generated by mice immunized by 100 µg of rhIFNγ mixed with FCA and those immunized with 100 µg of rhIFNγ and RIBI adjuvant, as shown in Figure 8a and b.

Discussion

In this study, the expression and purification of recombinant human interferon-gamma (rhIFN-γ) in *E. coli* were optimized. The study also compared adjuvant systems for generating humoral responses against rhIFN-γ. The major findings were: (i) optimal rhIFN-γ expression under induction with 0.6 mM IPTG, incubation temperature of 37°C, agitation speed of 250 rpm, and a 4-hour expression period; (ii) rhIFN-γ was mainly expressed in inclusion bodies; (III) denaturing purification of rhIFN-γ was maximised when the denaturing approach was used with careful imidazole titration; where low imidazole in the wash minimized contaminant retention while higher imidazole in the elution enhanced the recovery of the target protein; and (Iv) RIBI adjuvant produced same antibody level comparable to Freund's Complete Adjuvant (FCA) after boosting but with fewer local adverse reactions in our animal model.

Different organisms have different preferences for codon usage, meaning they may have preferred codons for the same amino acid.[27] When a gene from one organism is introduced into another, the codon usage of the gene may not match the codon usage preferences of the host organism. This mismatch can lead to inefficient translation of the gene into protein, reducing the expression levels or even leading to the production of non-functional proteins.[28] Codon optimization involves strategically modifying the nucleotide sequence of a gene to replace rare or less-favored codons with more frequently used codons in the host organism. This involves changing the synonymous codon of mRNA molecule while maintaining the encoded amino acid sequence.[29] By doing so, the efficiency of translation is increased, resulting in higher levels of protein expression.[30] A wide variety of factors regulate and influence gene expression levels, and GenSmart codon optimization tool takes into consideration as many of them as possible, but not limited to, codon usage, GC-content, mRNA secondary structure of the genes (eg., mRNA free energy), cis-acting mRNA destabilizing motifs, RNase splicing sites, and repetitive elements. Codon optimization improved the aptness of the human IFN-γ gene for expression in *E. coli*, increasing GC content from 39% to 44.71%, a range related to enhanced mRNA stability and translation efficiency.[31] Maintenance of the structural stability by the optimized codons was exhibited by the successful cloning of

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the IFN-γ gene into pET-28a(++), and the recovery of 13/14 positive colonies, consistent with findings in similar cytokine expression studies.[32] PCR validation of the expected 510 bp insert further supports correct construct assembly.[32] In general, the findings align with the literature, which shows that codon optimization improves heterologous protein expression in prokaryotic hosts.

The finding of optimal expression under induction with 0.6 mM IPTG, incubation at 37°C with shaking at 250 rpm for 4 hours, is in agreement with earlier studies that reported effective rhIFN-γ expression in lac/promoter-driven systems under similar conditions. Furthermore, insoluble product and inclusion body formation are enhanced by short, mid-log phase inductions at 37°C.[33] Brief induction periods of about 2 to 6 hours have been reported to give higher expression before the nutrients are depleted or proteolysis reduces yields, which is consistent with our finding, which found that expression declines after 4 hours, likely because of nutrient depletion and stress-related proteolysis.[34]

Protein expression conditions, such as temperature and agitation speed, are well acknowledged to shift the equilibrium between soluble and insoluble expression. Lower temperatures ranging between 16 to 25°C and gentle agitation of 120 to 200 rpm have been reported to enhance solubility by reducing the rate of translation; thereby, allowing correct folding, but at the expense of the growth rate and total yield, and this is a well-documented trade-off.[33] This explains why our attempt at overnight expression at 20°C and 150 rpm produced insufficient rhIFN-γ. This implies that lower temperatures improve solubility but reduce total protein yield, thus, not feasible when volumetric productivity is desired. This trade-off reinforces the large producers' approach of tolerating inclusion body formation and refolding rather than sacrificing yield.

The concentration of rhIFN-γ in inclusion bodies is an expected outcome of high-level expression in *E. coli* and is well explained in several recombinant cytokine and growth-factor expression studies.[35] Currently, inclusion bodies are treated as an appropriate concentrated reservoir of fundamentally pure polypeptide that can be solubilized and renatured; and success is subjected to solubilization approach, refolding ingredients, and strategies for the scale-up process.[36] For example, techniques including but not limited to detergent-assisted, chromatography refolding, dilution, diafiltration, urea chaperones, and urea-combined ammonium chloride have been developed to produce active soluble proteins.[37] In this study, the choice to use the denaturing purification approach followed by refolding aligns with established protocols.[38]

Imidazole's role in protein purification is to competitively elute histidine-tagged proteins from Immobilized Metal Affinity Chromatography columns by binding to the metal ions that hold the protein. It acts as a histidine analog, disrupting the strong interaction between the protein's polyhistidine tag and the metal on the resin.[39] The study found that low imidazole concentration in the wash buffers enhances purity by removing nonspecific binders; however, gradually increasing the imidazole concentration in the wash reduces the quantity of the recombinant protein purified, and this is in agreement with standard Nickel–Nitrilotriacetic Acid (Ni-NTA)/Immobilized Metal Affinity Chromatography (IMAC) practice.[40] Modest imidazole concentrations ranging from 10 to 50 mM in wash buffers intended to displace the weak nonspecific interactions are recommended by the manufacturers' manuals.[41] However, to maintain capture of His-tagged protein, and obtain higher recombinant concentrations, higher imidazole concentrations ranging between 200 and 400 mM in the elution buffer are endorsed.[42] Therefore, extreme imidazole concentration in the wash buffer can hastily elute the target protein, leading to low yield, whereas inadequate imidazole concentration fails to displace contaminants bound to the protein. Thus, our findings support the practical IMAC guidelines of titrating imidazole in minute increases to determine the optimal conciliation between purity and yield.[43]

Recombinant proteins such as rhIFNγ naturally provoke weak immune responses when administered unaided, requiring the use of adjuvants to augment immunogenicity in model animals.[44] In this study, we evaluated the immune-potentiating effects of three commonly used adjuvants: Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), and RIBI adjuvant on relative antibody levels as an indicator of immunogenicity following vaccination with rhIFNγ. Overall, the findings confirmed that the choice of adjuvant strongly influenced both the magnitude of the antibody response and the occurrence of adverse effects.

Among the three adjuvants tested, FCA produced the highest relative antibody levels, consistent with its well-documented potency. However, this potent immune incitement was accompanied by observable side effects, such as swelling, soreness, formation of humps, and scarring at the injection site, particularly when 100 µg of rhIFNγ was administered with an equal volume of FCA.[45] These adverse inflammation effects underline the limitations of FCA, despite its greater immune-augmenting effects, and align with previous studies that reported its adverse side effects.[46] FIA elicited a weaker initial immune response, thus confirming its lower efficacy compared to FCA. However, the findings also revealed that recurring boosting markedly enhanced the relative antibody levels in the FIA-vaccinated group. This implies that when a study prioritizes minimizing animal discomfort, FIA is a viable alternative for antibody production, with multiple boosts. Furthermore, FCA was anticipated to outdo all other adjuvants; however, the immunogenic potential between FCA (100 μg rhIFNγ) and RIBI adjuvant (100 μg rhIFNγ) after two boosts was not statistically significant. Thus, RIBI adjuvant elicited relative antibody responses comparable to FCA but with substantially fewer side effects. This finding underlines RIBI as a safer and efficacious choice for high-quality antibody production, especially in research settings where animal welfare is prioritized.

In summary, the findings of this study reveal that FCA retains its position as the most efficacious adjuvant in eliciting the immune response; however, its associated adverse inflammatory reactions hinder its routine laboratory use. Nevertheless, RIBI adjuvant offers a pronounced balance between safety and high immunogenicity, making it the preferred choice based on this study. This study recommends adjusting the dosage to improve tolerability for studies that still elect to use Freund's adjuvants: 25 μg of rhIFNγ mixed with an equal amount of FCA with multiple boosts, or 50 μg of the protein mixed with an equal volume of FIA, depending on the required antibody titres and tolerable levels of inflammation.

Limitations

Despite the successful expression and purification of recombinant Interferon gamma, the study had several limitations. Although the protein identity was confirmed using SDS-PAGE and Western blot analysis, the present study did not include an in vitro bioactivity assay to directly demonstrate the biological functionality of the recombinant protein. Functional assays, such as macrophage activation and antiviral activity assays, would provide additional confirmation of the biological activity of the expressed protein. Also, endotoxin removal and quantification were not performed following purification of the recombinant protein expressed in *Escherichia coli*. Since endotoxin contamination can occur during recombinant protein production in bacterial systems, future studies should incorporate endotoxin removal procedures and quantification methods such as the Limulus Amebocyte Lysate assay to further validate the suitability of the recombinant protein for downstream biological applications.

Conclusion

The study demonstrated that protein expression yield is affected by time, temperature, and imidazole acts as a competitive elution agent to remove contaminants in purification. Although FCA has been reported to produce the highest antibody titer with mild to severe side effects, in our study, there was no significant difference between the relative antibody levels generated from the group of mice immunized with 100 μg of rhIFNγ mixed with FCA and the group immunized with 100 μg of rhIFNγ mixed with RIBI adjuvant.

Data Sharing Statement

Data associated with this study have been incorporated into this manuscript.

Ethical Consideration

Ethical clearance with Reference Number SVAR-IACUC/195/2024 was obtained from the Institutional Animal Care and Use Committee (IACUC), College of Veterinary, Animal Resources, and Biosecurity (COVAB), Makerere University. The animals were treated humanely to avoid any discomfort, such as excessive pain and stress. The study adhered to the 3Rs principles of Reduce, Refine, and Replace to ensure the use of the fewest number of animals possible according to the Organization for Economic Cooperation and Development (OECD) protocols.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests.

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