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Characterization And Optimization Of Recombinant Gm-Csf Protein: Expression And Functional Analysis

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Abstract

Background: Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) emerges as a key influencer, wielding its impact over haematopoiesis and immune modulation. It can address an array of intricate physiological and pathological scenarios.

Objective: The present study is aimed to characterize and optimize the recombinant GM-CSF protein and to carry out its expression and functional analysis.

Methods: GS115 (Pichia pastoris) strain was double digested with restriction enzymes 5'AOX1 and 3'AOX1α and ligated to the pPICZα vector. The positive clones were screened using PCR, mobility shift, Kex2 signal cleavage and restriction digestion. Further, the Pichia strain with pPICZα A was transformed into yeast cells, and its expression was studied using Spectroscopy and SDS-PAGE. In addition, the resulting Protein was purified using reverse-phase column chromatography and functional characterization was performed using a Pichia pastoris HCP kit.

Results: The GM-CSF protein was successfully transformed into yeast cells, andtheSDS-PAGE profile confirmed the presence of GM-CSF in the expression system. A purified form of recombinant GM-CSF protein was obtained using HPLC, and the obtained chromatograms of both reference and test samples were comparable. Further, from the functional analysis, about9.67 ppm of the functional hematopoietic cell phosphatase (HCP) was observed in the purified GM-CSF sample.

Conclusion: The Recombinant GM-CSF has been successfully prepared and confirmed for their expression and functional characteristics. The developed Protein can regulate production, cell differentiation and granulocytic functions.

Keywords: Recombinant GM-CSF, Pichia pastoris, pPICZα vector, Hematopoietic cell phosphatase, Expression analysis.

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a specific factor stimulating the proliferation of many hematopoietic cells like granulocytes, monocytes and macrophage progenitors to form matured colonies of granulocytes and macrophages(Burgess &Metcalf, 1980). T and B cells, monocyte/macrophage endothelial cells, and fibroblasts are the leading producers of GM-CSF, which tumour cells, chondrocytes, path cells, eosinophils, epithelial cells, mesothelial cells, neutrophils can also produce. GM-CSF can stimulate dendritic cell (DC) maturation and the survival and activation of neutrophils, eosinophils, and macrophages invitro(Hamilton, 2002).More traits of a homeostatic cytokine, which typically controls the generation and homing of many myeloid cells, may be seen in M-CSF(Ushach&Zlotnik, 2016).GM-CSF plays a proinflammatory influence on myeloid cells and is produced in significant quantities by immune system cells that have become activated under inflammatory situations, whichinfluences myeloid cell activation and is a more viable target for the synthesis of medications to manage inflammatory autoimmune illnesses(Ushach&Zlotnik, 2016). All responsive hematopoietic lineage cells, as well as responsive and unresponsive nonhematopoietic cells, have been shown to have unique GM-CSF receptors(Baldwin, 1992).

The GM-CSF gene is situated directly downstream of the IL-3 gene in both humans and mice, where, within a tight locus spanning approximately 30 kb, the human GM-CSF and IL-3 genes and other related regulatory elements (3–11) are located, just 10 kb apart(Cockerill et al., 1999). A regulatory element containing two symmetrically nested inverted repeats has been discovered in the promoter of the GM-CSF gene(Cousins et al., 1998). GM-CSF is expressed in various cell types, including T cells, in response to immune or inflammatory stimuli. The induction of GM-CSF gene expression is regulated by a proximal promoter limited to 100 bp upstream of the transcription start site and an upstream enhancer. Induction of GM-CSF gene expression upon T cell activation is accompanied by changes in chromatin structure across the GM-CSF promoter. The chromatin remodelling events precede GM-CSF gene transcription and, similarly to gene transcription, depend on the presence of NF-kB protein (Brettingham-Moore et al., n.d.). Human GM-CSF comprises 127 amino acids and possesses two possible N-glycosylation sites and multiple O-glycosylation sites(Okamoto et al., 1991). The diversity in glycosylation leads to a broad range of apparent molecular weights for GM-CSF, ranging from 14.5 to 32 kDa(Kaushansky et al., 1992). The level of glycosylation of GM-CSF can impact its biological activity, pharmacokinetics, immunogenicity, and toxicity(Cebon$# et al., 1990; Wadhwa et al., 1996).

Presently, two preparations of recombinant GM-CSF, Sargramostim and Molgramostim, are available in the pharmaceutical market. These preparations consist of recombinant GM-CSF derived from Saccharomyces cerevisiae yeast and Escherichia coli bacteria. Molgramostim consists of GM-CSF that is not glycosylated and has an extra methionine at the N-terminal. The utilization of this cytokine is accompanied by autoimmune reactions and a significant occurrence of side effects, which were not approved by the FDA for therapeutic usage in the United States(Ragnhammar et al., 1994). Sargramostim consists of fully developed, glycosylated GM-CSF and has received approval from the FDA for clinical use.

Nevertheless, a drawback of utilizing S. cerevisiae yeast as a producer of therapeutic proteins is the production of hyperglycosylated proteins, which contain oligosaccharides with over 50 mannose residues, decreasing their biological activity(Ballou, 1990). Pichia pastoris, a type of yeast that uses methanol as a carbon source, offers several benefits for producing therapeutic proteins. These include its ability to perform posttranslational protein modifications that are similar to those found in humans, its lack of exo- and endotoxin production, its high yield of recombinant proteins, and the ease of purifying these proteins due to their secretion into the culture medium(Cereghino&Cregg, 2000).

Based on the above background, the present study aims to prepare and optimize recombinant GM-CSF using Pichia pastoris yeast and its characterization using expression and functional analysis.

Methodology

Procurement of Samples

Strains Used in this study: GS115 (Pichia pastoris)(Genotype *His4*; Application used: Selection of expression vector containing His4) was used as a host strain for this study,pPICZα A, B, C was used as a vector, and EasySelect™ Pichia Expression Kit was procured from Thermo Fischer.

Human GM-CSF gene construct using pPICZα

YPD media was used for growing the procured Pichia pastoris strain, where 1L of YPD contained 2% glucose, 2% peptone, 1% yeast extract, and 2.4% agar.The genomic DNA was isolated from overnight culture according to the modified protocol of Wright *et al.,* 2017. A brief description of the method includes about 2ml of bacterial culture being harvested by centrifugation, and the pellet was dissolved in lysozyme (500 µl), which was then incubated at 37 ^{0}c for 45 mins. About 100 µl of SDS (10 %) and 200 µl of NaCl were then added, and the mixture was incubated for an hour. The DNA was extracted from this bacterial cell lysate by adding phenol chloroform. Further, the isolated DNA was precipitated with 70 % ethanol and dissolved in 50 µl TE buffer. The DNA's integrity, purity, and concentrations were confirmed using agarose gel electrophoresis and checking the OD at 260/280 nm in the nanodrop (Thermo et al. USA).

Construction of the expression plasmid pPICZ α-GM-CSF-Apo A1

The hGMCSF gene was amplified by polymerase chain reaction (PCR). The amplified PCR signal sequence of 5'AOX1 and 3'AOX1α was double digested with Xho I and EcoR I and ligated to the pPICZα vector digested with restriction enzymes. The positive clones were screened by PCR, mobility shift, Kex2 signal cleavage and restriction digestion. The Human GM-CSF gene was PCR amplified from pPICZα-GM-Apo-A1 using primers P3 (forward) and P4 (reverse) having Xho I and EcoR I restriction sites, respectively.

Table 1-Primers used in the study

Primer	Sequence	Target
P1	5' GGA GGT AAC ATA TGT TTA AGT TTA AAA AG 3'	AOX1 α-factor
P2	5' GGC CAG CTG CAG AGG CGG TTG CCG AAA AC 3'	
P3	5' GGC CAG CTG CAG GCA CCC GCC CGC TCG 3'	Pst I
P4	5' C GAA TTC TCA CTC CTG GAC TGG 3'	EcoR I

Transformation of Yeast Cells

Pichia strain was transformed using the Pichia EasyComp™ Transformation Kit (Source: Invitrogen) with pPICZα A. Competent cellswere prepared and yield optimized.Further restriction Digestion was performed using Sac1 enzyme in ~5–10 µg of plasmid DNA, and a small aliquot was observed under agarose gel electrophoresis for complete linearization. Once the vector was found to be completely linearized, the reaction was then heat-inactivated, and EDTA was added to stop the reaction. About 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol were then added to the above reaction mixture to precipitate the transformed DNA, and the pellet was further washed with 80% ethanol, air-dried, and resuspended in 10 µl sterile, deionized water.Post transformation, the obtained transformed cells were tested on YPDS plates containing 50µg/mL of Zeocin.

Optimization of Expression in Shake Flask (By OD at 600nm Spectroscopy method and SDS-PAGE profile characterization)- Post Induction Harvest

The expression of rhGM-CSF was assessed in shake flasks by inoculating a single colony inoculated in 20 mL of BMGY medium in a 100 mL Shake flask and incubated at 28–30°C in a shaking incubator (250–300 rpm) until the culture reached OD_{600} = 2–6 log-phase growth, approximately 16–18 hours. The cell growth was then monitored at OD600 nm, and the cells were harvested by centrifugating at 1,000 × g for 5 minutes at 4°C. The cell suspension was then resuspended in BMMY medium to 1.0 at OD600 in 20 mLto induce expression. About100-200µl of 100% methanol was added directly to culture flasks to reach a final concentration of 0.5-1%. The culture was then placed in a 100 mL flask and further incubatedfor continuing growth in an orbital shaker incubator at 250-300 rpm. 100% methanol was added to the above flaskevery 24 hours to make a final concentration of 0.5-1% to maintain induction for up to 3 days.

Optimization and characterization of the simplified purification strategy

The clarified culture was harvested, and the pellet was dissolved in 20 mM sodium acetate and 0.5 mM EDTA at pH 4.0.Dialysis was performed using a 20K MWCO membrane, and the resultant was loaded to Reverse Phase column chromatography on a 50 ml column packed with Source-30 RPC matrix for purification. The column and the buffers (Buffer-A: 0.1% TFA in distilled water and Buffer-B: 0.1% TFA in Acetonitrile) were connected and equilibrated using the above buffers by pumping at a 5 ml/min flow

rate. The fractions for Loaf, flow material, wash1 & 2 and elute 1,2 &3 were then collected and were subjected to protein characterization by Physico-chemical methods.

Physico-chemical characterization of purified recombinant rhGM-CSF

Protein Quantification by BCA method:

The purified GM-CSF samples and Bovine serum albumin (BSA) (Sigma-Aldrich) were quantified by the BCA method, OD at 562 nm, based on the extinction coefficient of BSA and GM-CSF. The stock solutions were diluted in distilled water (1/10, 1/20, and 1/ 50) to obtain different Protein concentrations. The assays were carried out in triplicates, and the regression equation for the standard curve was calculated using MS Excel. OD for 1/20 and 1/ 50 dilutions obtained were very low. Hence, 1/10 dilution was considered for calculating total Protein.The expression of hGMCSF was analyzed using SDS-PAGE, and Western blot analysis was done using anti-human GM-CSF monoclonal antibodies.

Chromatographic Purity profile by SE-HPLC

GM-CSF standard (Commercially available) and purified GM-CSF were diluted in mobile phase solution (12.8 g sodium dihydrogen phosphate, 19.1 g disodium hydrogen phosphate, 50 ml of ethanol and qs to 1 L with distilled water, pH 6.8) to obtain the final concentration 0.4 mg/ml. HPLC system (Water's) with refractive index detector (RID) consisting the Shodex protein KW-G Guard column (6.0 mm X 50 mm), Shodex protein KW803 (8.0 mm x 300 mm, five µm) and Shodex protein KW804 (8.0 mm x 300 mm, seven µm), with a flow rate of 0.5 ml/min, 25°C column temperature, injection volume of 30 µl, and 60 min run time. The purity and retention time of both standard and purified GM-CSF were compared.

Optimization

Size Exclusion-HPLC for the final concentrate protein was performed by optimizing different incubation conditions and diluent to assess the presence of High Molecular Weight protein and charge variants in the concentrate. Various incubation conditions and diluent for resolution were prepared to study the protein aggregates.

Incubation Condition: 1mg/mL sample solution incubated up to 90º for 120 minutes.

Samples were collected in various incubation times, i.e., 10, 20, 40, 50, 55, 60, 75 and 90º

Diluent: 10 mM L-glutamic acid, 4% mannitol, 2% sucrose, 0.01% polysorbate 20, pH 4.0

Peptide Mapping of the final sample and for Amino Acid position confirmation by HPLC

Sample Preparation: 50 µl of 0.05 M sodium phosphate buffer, pH 8.0, was dispensed in two 1.5 ml polypropylene tubes labelled as test and reference. The volume of the test solution and reference solution corresponding to 25 µg of Protein was added to each tube. 25 µl of glutamyl endopeptidase (0.1 mg/ml solution, Roche) was added to both tubes and made up to 1 ml with water. These tubes were mixed and incubated in a water bath at 37° C for 18 hrs, and 125 µl of guanidine hydrochloride (8 M) and ten µl of DTT (1 M) solutions were added at the end of the incubation and further incubated in boiling water bath for 1 minute and cooled to room temperature.

Chromatography

YMC column, which is 100 mm long and 2.1 mm in internal diameter packed with octadecylsilyl silicagel (5 µm), 20 nm pore sizewas attached to Dionex HPLC system with UV detector (VWD/PDA) (Dionex Ultimate 3000). The column was equilibrated with 97 % mobile phase A (5 % Acetonitrile) and 3 % mobile phase B (95 % Acetonitrile) at a flow rate of 0.2 ml/minute at 60°C for 30 mins and then µl of sample and reference sample was loaded on the HPLC column. Gradient elution was performedwith a flow rate of 0.2 ml /min at 600C and a run time of 65 mins. The chromatograms obtained for the test samples were compared with reference samples using software (Chromeleon).

Table 2: Program for separating peptide fragments on HPLC column with UV detector.

Time (min.)	Flow rate (ml/min.)	Mobile phase A (% V/V)	Mobile phase B (% V/V)
0	0.2	97	3
8	0.2	94	6
25	0.2	66	34
40	0.2	10	90
45	0.2	10	90
46	0.2	97	3

65	0.2	97	3

Functional characterization and Biological activity of Purified recombinant rhGM-CSF
Host Cell Protein (HCP)
The residual host cell protein in purified GM-CSF sample was determined using a Pichia pastoris HCP kit (Cygnus Technology. Inc, USA) according to the manufacturer's instructions. The concentration of residual HCP was extrapolated from the standard curve generated from known concentrations of standard HCP (provided with the kit).

Results

Expression vector construction and transformation studies
TheKex2 gene was successfully incorporated into plasmid vector pPICZ α-GM-CSF-ApoA1 constructed upon transformation and was confirmed by PCR, which yielded two bands of DNA (2kb and 3.5 -4kb) upon restriction digestion. The PCR product of 400 bp revealed a positive reaction for AOX and a sequence of 788 bp. The obtained sequence is mentioned in Table 2 and Figure 1.

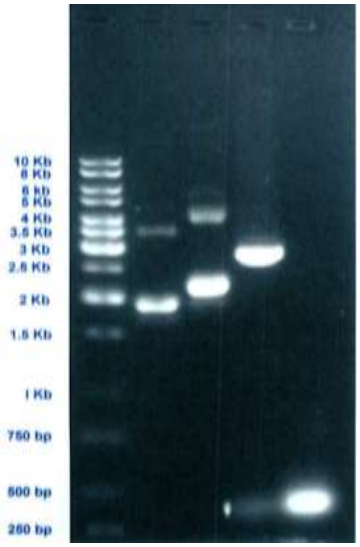

Fig.1-Lane I: DNA molecular weight marker (10 Kb ladder), 2,3: pPICZα A plasmid (un-digested), 4,5: pPICZα A plasmid digested with AOX 1 & AOX 2 and 6: PCR amplified product of pPICZα A-GM-CSF-Apo1 construct, Human GM-CSF gene.

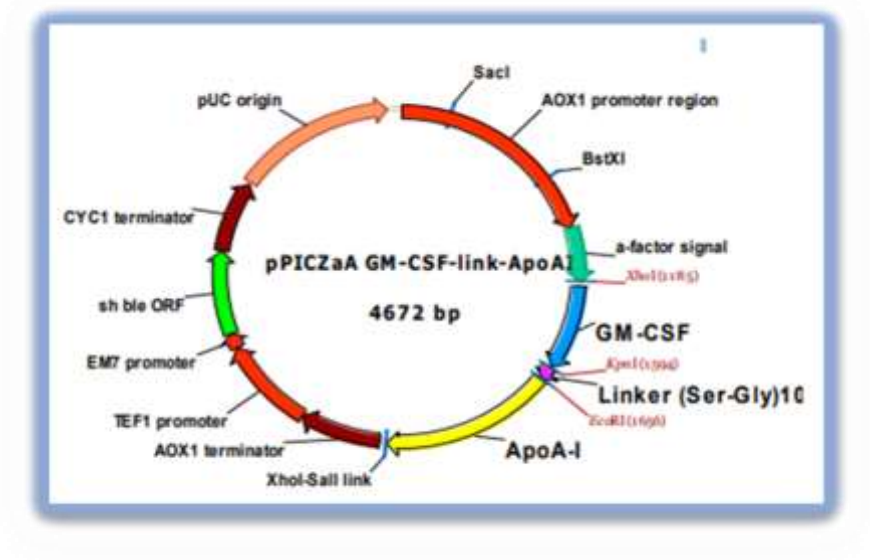

Fig.2-Constructed pPICZ α-GM-CSF-ApoA1 plasmid

Gene Sequence

1	AGTACACAGA	GAGAAAGGCT	AAAGTTCTCT	GGAGGATGTG	GCTGCAGAGC
51	CTGCTGCTCT	TGGGCACTGT	GGCCTGCAGC	ATCTCTGCAC	CCGCCCGCTC
101	GCCCAGCCCC	AGCACGCAGC	CCTGGGAGCA	TGTGAATGCC	ATCCAGGAGG
151	CCCGGCGTCT	CCTGAACCTG	AGTAGAGACA	CTGCTGCTGA	GATGAATGAA
201	ACAGTAGAAG	TCATCTCAGA	AATGTTTGAC	CTCCAGGAGC	CGACCTGCCT
251	ACAGACCCGC	CTGGAGCTGT	ACAAGCAGGG	CCTGCGGGGC	AGCCTCACCA
301	AGCTCAAGGG	CCCCTTGACC	ATGATGGCCA	GCCACTACAA	GCAGCACTGC
351	CCTCCAACCC	CGGAAACTTC	CTGTGCAACC	CAGATTATCA	CCTTTGAAAG
401	TTTCAAAGAG	AACCTGAAGG	ACTTTCTGCT	TGTCATCCCC	TTTGACTGCT
451	GGGAGCCAGT	CCAGGAGTGA	GACCGGCCAG	ATGAGGCTGG	CCAAGCCGGG
501	GAGCTGCTCT	CTCATGAAAC	AAGAGCTAGA	AACTCAGGAT	GGTCATCTTG
551	GAGGGACCAA	GGGGTGGGCC	ACAGCCATGG	TGGGAGTGGC	CTGGACCTGC
601	CCTGGGCCAC	ACTGACCCTG	ATACAGGCAT	GGCAGAAGAA	TGGGAATATT
651	TTATACTGAC	AGAAATCAGT	AATATTTATA	TATTTATATT	TTTAAAATAT
701	TTATTTATTT	ATTTATTTAA	GTTCATATTC	CATATTTATT	CAAGATGTTT
751	TACCGTAATA	ATTATTATTA	AAAATATGCT	TCTACTTG	

Optimization of Expression in Shake Flask

OD at 600 values resulted in the Protein build-up inside the cells and SDS-PAGE profile confirmed the presence of GM-CSF product in expression system which started increasing tremendously after 48 hrs of methanol induction, which began to increase by three times in 72hrs.The Post 72 Hrs expression harvest subjected for SDS-PAGE to optimize and assess induction revealed that the band at18 kDa confirmed the expression corresponding to human GM-CSF.

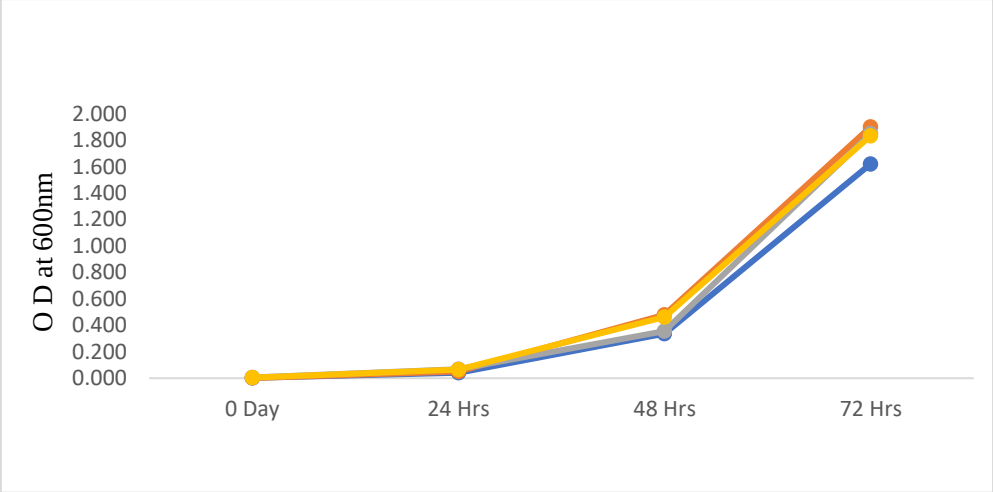

Fig.3- Methanol-Induced Expression of Pichia Pastoris Expressed GM-CSF

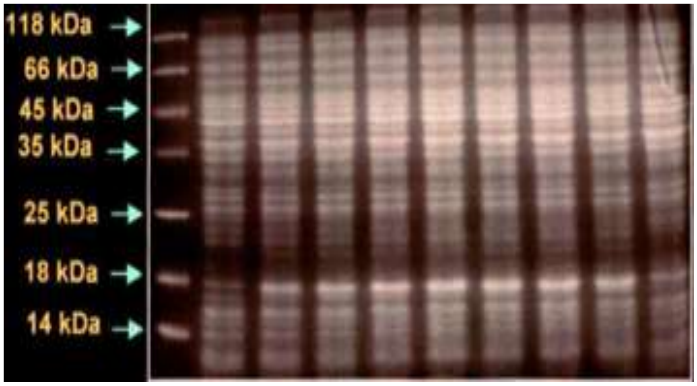

Fig.4-The Post 72 Hrs expression harvest subjected to SDS-PAGE to optimize and assess induction

Protein characterization by Physico-chemical methods

Upon plotting the BSA at different concentrations against OD at 562 nm, the obtained concentration was 400mg/mL for the sample protein, and the total Protein was 2.3 mg/mL. The SDS-PAGE profile of multiple fractions and final fraction confirmed the presence of the GM-CSF—the final concentrated

Protein identified on a western blot using anti-human GM-CSF monoclonal antibodies. The GM-CSF final concentrated protein was identified using anti-human GM-CSF monoclonal antibodies on a western blot. A single band of approximately 18 kDa corresponding to human GM-CSF was confirmed.

Table 3-Optimization and characterization of the simplified purification strategy

Sample	Conc (µg/mL)	OD at 562 nm
BSA	40	0.9985
	20	0.498
	10	0.251
	5	0.124
	2.5	0.0635
	1	0.00355
	0.5	0.001795
Sample (GM-CSF Concentrate)	1/ 10 Diluted	1.3934
Sample Protein (µg/mL)	400	
Total Protein (mg/mL)	2.3	

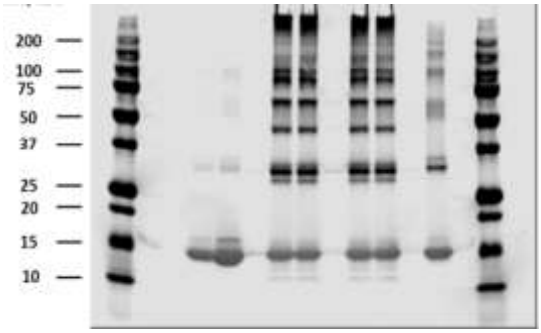

Fig.5-SDS-PAGE Profile of multiple fraction elution

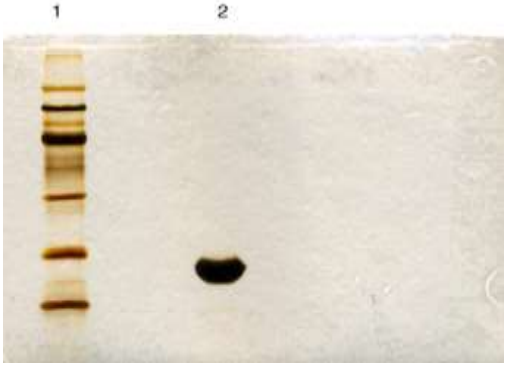

Fig.6-Lane 2 was loaded with the final concentrate of GM-CSF (10 µg). Protein low molecular weight marker (Bio-rad) was loaded in Lane 1.

Chromatographic Purity profile by SE-HPLC

The retention time of the primary sample was 29.311 with 98.325% of area Fig.7 and Table. An average of 9.2% aggregate area, 4.3% dimers, and 86.5% monomers were observed in the final concentrated protein sample. The sample was found to be stable up to 60º incubation condition. The highest average aggregate % of area was found to be 86.5%. Eight significant peaks were observed for AA position confirmation, corresponding to the standard product peaks and amino acid positions. The amino acid positions and retention times for the peaks arementioned in Table 3.

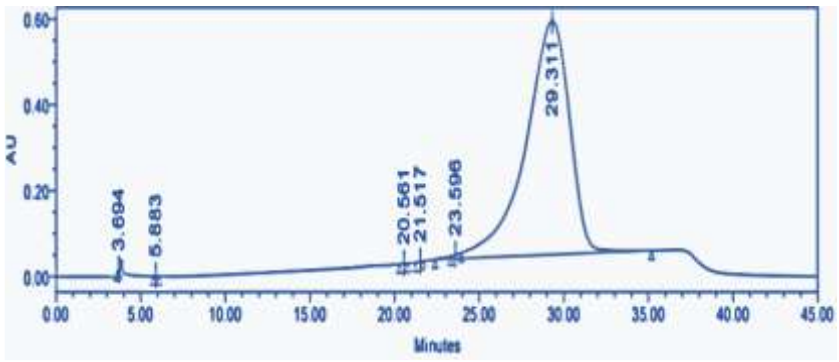

Fig.7- chromatogram showing the purity profile of the rhGM-CSFsample

Table 4-Purity profile of the rhGM-CSFprotein sample

Peak	RT	Area	%Area
Unknown	3.694	4944	0.0160
Unknown	5.883	9844	0.0312
Oxidized	20.561	138721	0.4236
Oxidized	21.517	159823	0.5169
Reduced	23.596	190764	0.6873
Main Sample	29.311	31326795	98.325

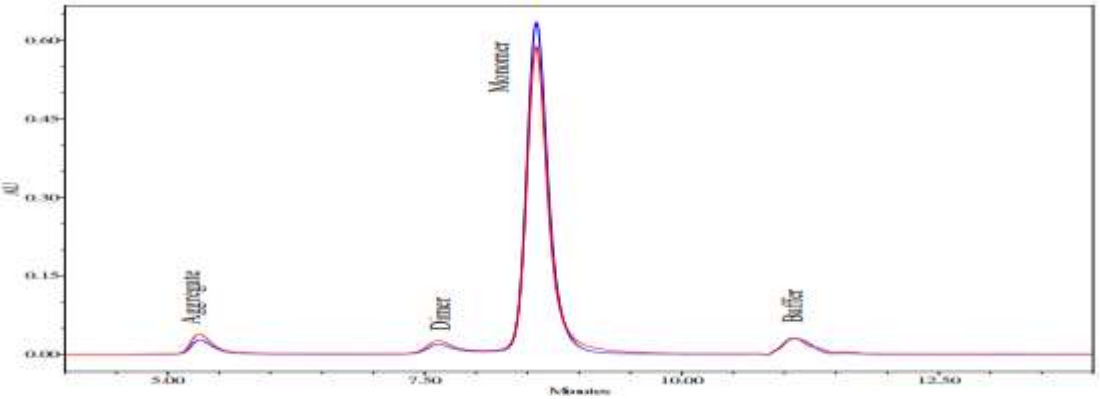

Fig.9-Chromatogram showing aggregate area distribution of different compounds in the rhGM-CSF Protein

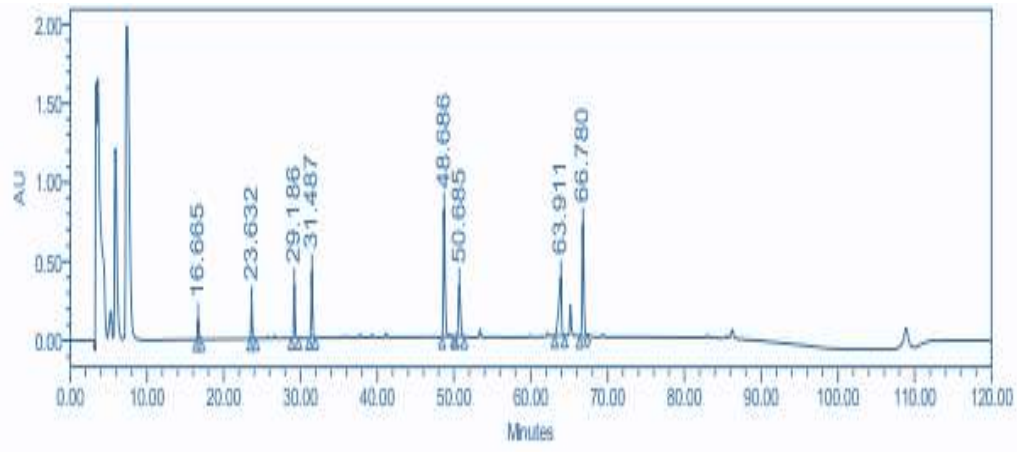

Fig.8-Chromatogram showing the amino acid position of the rhGM-CSF protein

Table 5-Amino acid position of the rhGM-CSF protein

Peak	Amino Acid Position	RT (Minutes)
1	95-99	16.7
2	21-34	23.6
3	35-47	29.2
4	164-175	31.5
5	125-163	48.7
6	1-20	50.7
7	100-124	64.0
8	48-94	66.8

Functional characterization and Biological activity of Purified recombinant rhGM-CSF

The concentration of residual HCP was extrapolated from the standard curve generated from known concentrations of standard HCP (provided with the kit). The calculated concentration of HCP in the purified GM-CSF sample was 9.67 ppm.

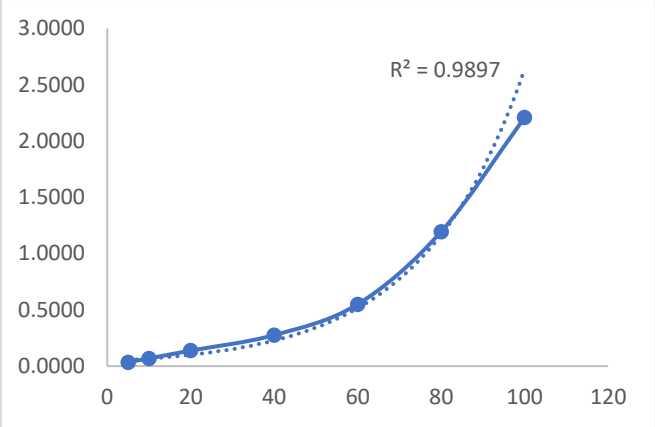

Fig.9-Standard curve for determining residual host cell protein in purified GM-CSF

Discussion

The majority of biotherapeutics currently available on the market (over 50%) are manufactured using mammalian cell lines despite the existence of other platforms such as bacteria, yeasts, and plant cells. The requirement for precise posttranslational alterations drives this preference for mammalian cell lines. The trait of glycosylation is highly consequential as glycans, when attached to proteins, impact biological activity, half-life, and tissue distribution and occasionally exhibit immunogenicity.Twenty-three GMCSF is a biotherapeutic belonging to the colony-stimulating factors family and is renowned for influencing the differentiation and proliferation of hematopoietic cells(Alexander et al., 1998). Despite numerous attempts, researchers are currently focused on employing E. coli and yeast systems to create rhGM-CSF. GMCSF is more active in its non-glycosylated form, providing an advantage over other expression hosts. A comparative investigation was undertaken in the current trial to analyze the productivity and activity of cytosolic and secretory expression of rhGM-CSF, which possesses an N-terminal fusion of the intein-chitin-binding domain. The P. pastoris GS115 host system utilized pPICZ to express the expression.

The conventional AOX-based vector was altered to incorporate the intein-CBD, and the expression cassette was incorporated into the P. pastoris GS115 host. The multi-copy integrants were screened using escalating doses of zeocin. A three-stage high-cell density cultivation method in P. pastoris often achieves heterologous protein expression. The current approach employed to express this Protein results in a yield of up to 400 mg/L through intracellular expression using the Pichia GS115 strain. Similar expression investigations utilized P. pastoris SMD1168 to compare E. coli BL21 (DE3) and E. coli GJ1158. Pal et al.,2006, observed the extracellular protein expression at a concentration of 250 mg/L, where the toxicity of the Protein was significantly reduced. The glycoengineered P. pastoris strain was utilized to produce granulocyte–macrophage colony-stimulating factor. It was discovered that GMCSF was generated at elevated concentrations (hundreds of milligrams per litre)(Jacobs et al., 2010) when a traditional purifying technique was employed. The protein yield obtained from P. pastoris GS115 was more significant than those achieved using E. coli and P. pastoris SMD1168-based expression systems, as documented in other publications(Babu et al., 2008,2009).

The production of GM-CSF in this study was lower than that of murine granulocyte-macrophage colony-stimulating factor in Pichia. However, using an intein tag to manufacture hGMCSF can reduce production costs and simplify the purification of this biotherapeutic drug on a broader scale. Traditionally, histidine ligand affinity chromatography has been utilized to purify proteins (Kaur &Reinhardt, 2012). Subsequently, intein-based systems incorporating a chitin-binding domain were devised to address the two-step purification process and the costly enzymatic cleavage of fusion proteins.Other histidine or GST-based fusion protein purification techniques have superseded this approach due to nonspecific protein binding, costly enzymatic cleavage, and the need for further enzyme purification. The intein tag purification process stands out from other purification methods due to its unique capability to purify a substance in a single chromatographic stage while achieving high yield and maintaining activity 27 and 28. Additional fermentation experiments can optimize output and provide a cost-effective method for the mass production of this vital biotherapeutic.This study presents a detailed account of the efficient production and release of mGM-CSF in P. pastoris. This was achieved by utilizing the AOX1 gene promoter with the S. cerevisiae α-mating factor secretion signal. Previous research has shown that mGM-CSF may be expressed in various types of cells, including transfected COS cells, genetically modified plant cells, E. coli, and S. cerevisiae. However, the expression levels in these cells were low. In order to conduct large in vivo experiments, it is necessary to get milligram quantities of highly purified mGM-CSF. By utilizing a meticulously regulated fermentor to cultivate P. pastoris, a substantial level of mGMCSF expression and secretion was attained.

Furthermore, a technique was employed for modifying proteins using amine-targeted PEGylation, employing gentle coupling conditions, resulting in an average of 2-3 sites of conjugation per molecule. The final outcome is a highly purified tiny bioactive chemical that strongly promotes the growth of myeloid progenitor cells originating from bone marrow. These advancements allow for more extensive studies on the specific cellular and molecular processes that control inflammation in the gut mucosa.

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