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(54) Title: NOVEL CLONING, EXPRESSION & PURIFICATION METHOD FOR THE PREPARATION OF RANIBIZUMAB

(57) Abstract: The present invention relates to Ranibizumab cloning, expression and production using a novel approach for better yield and biologically active protein.

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RELATED APPLICATIONS

This application is related to Indian Provisional Application 1570/MUM/2013 filed 30th Apr, 2013 and is incorporated herein in its entirety.

FIELD OF THE INVENTION

The present invention relates to Ranibizumab cloning, expression and production using a novel approach for better yield and biologically active protein.

BACKGROUND OF THE INVENTION

Proteins are important as these are used to cure a number of diseases including angiogenesis (VEGF) diabetes (e.g. Insulin), cancers (e.g. Interferon, monoclonal antibodies), heart attacks, strokes, cystic fibrosis (e.g. Enzymes, Blood factors), inflammation diseases (e.g. Tumor Necrosis Factors), anemia (e.g. Erythropoietin), hemophilia (e.g. Blood clotting factors) etc. One of the important challenges is the development of efficient and competent process for the cloning, expression and large scale purification of these proteins. Numerous processes are available for the cloning, expression and large scale purification of desired protein from the cell culture supernatants, but still it is difficult to clone, express and separate the desired protein from the mixture.

Vascular endothelial growth factor A (VEGF-A) is a biological component that can trigger angiogenesis, which is the growth of new blood vessels. Various diseases, inter alia, ischemia, anemia, peripheral vascular disease, and atherosclerotic lesions can be treated by increasing angiogenesis. This is accomplished by stimulating the up-regulation of VEGF-A, thereby leading to increased blood circulation, hence increased oxygen supply, in the diseased tissue. In the eye, however, excessive vascularization can result in blood and fluid leaking into the eye. These leaky blood vessels can contribute to macular edema and choroidal neovascularization, resulting in the wet type of age-related macular degeneration (AMD). The result of AMD can be the loss of visual acuity or even blindness. Therefore, control of excessive macular vascularization is important in the treatment of macular degeneration. As such, it is a goal of medical professionals to provide a treatment for controlling or curing AMD without inhibiting the beneficial effects of normal VEGF-A activity in the rest of the body. Ranibizumab has been found to be an effective treatment of AMD.

Ranibizumab is a recombinant humanized IgGI kappa isotype monoclonal antibody that inhibits VEGF activity by competitively binding to the receptor binding site of active forms of VEGF-A, including the biologically active, cleaved form of this molecule, VEGF110. Hence, Ranibizumab prevents binding of VEGF-A to its principle receptors VEGFR1 and VEGFR2 found on the surface of endothelial cells. This results in reduced endothelial cell proliferation, vascular leakage, and new blood vessel formation.

LUCENTIS® is a medical formulation of Ranibizumab and designed for intraocular injection directly into the vitreous humor of the eye, wherein the active ingredient Ranibizumab penetrates the internal limiting membrane to access the subretinal space. These injections are typically given from 5 to 7 times a year to patients and in many instances are given monthly. Since the cost of treatment is very high and not many patients can afford it as such, there is still a need for improved cloning process and purification of the protein so that the production efficiency can be enhanced and the drug can be produced cheaper.

US6998383 discloses RANK inhibitor consisting of a TRAF-6 binding domain attached to a leader sequence.

US7569384 discloses nucleic acid molecule encoding albumin fusion protein. Also discloses vectors containing these nucleic acids, host cell transformed with these nucleic acid vectors and methods of making the albumin fusion protein using these nucleic acids, vectors and/or host cell.

US8597907 discloses a method for efficiently producing an industrially useful protein in coryneform bacteria. The present invention provides a method for efficiently producing heterologous proteins comprising culturing coryneform bacteria containing an genetic construction containing a promoter sequence which functions in coryneform bacteria, a nucleic acid sequence encoding a Tat system-dependent signal peptide region, and a nucleic acid sequence encoding a heterologous protein, in the direction from 5'-end to 3'-end, and secretory producing the heterologous protein by coryneform bacteria.

US5641870 discloses a process for purifying an antibody. In this process, a mixture containing the antibody and contaminant is subjected to low pH hydrophobic interaction chromatography (LPHIC) optionally at low salt concentration. The antibody is eluted from the column in the fraction which does not bind thereto. This process can be preceded and followed by other purification steps.

US7847071 discloses a method of purifying an antibody comprising the steps of: firstly, purifying an antibody by means of protein A affinity chromatography wherein the protein A is a native protein A or a functional derivative thereof, secondly, loading the purified antibody comprising a protein A-

contaminant, wherein said protein A-contaminant is obtained upon eluting bound antibody from said protein A affinity chromatography, on a first ion exchanger under conditions which allow for binding of the protein A or its derivative, thirdly, collecting the antibody loaded onto the first ion exchanger in the flow-through of the first ion exchanger whilst a contaminant protein A is bound to the first ion exchanger, wherein the first ion exchanger is an anion exchanger, further purifying the antibody by loading on, binding to and eluting it from a second ion exchanger, and discarding a tail fraction of the eluate of the second ion exchanger such that a monomeric antibody fraction is enriched as a purified antibody pool.

WO2011089212 discloses a method for depleting impurities, in particular host cell proteins (HCP) and DNA from cell culture supernatants by means of protein A chromatography using a novel washing buffer.

Liu et al., discussed in mAbs 2, 2010, 480-499 about the basic unit operations such as harvest, Protein A affinity chromatography and additional polishing steps along with alternative processes such as flocculation, precipitation and membrane chromatography and also covered platform approaches to purification methods development, use of high throughput screening methods, and offered a view on future developments in purification methodology as applied to monoclonal antibodies.

Refolding of inclusion body proteins into bioactive forms is cumbersome, requires many operational steps and most of the time results in very low recovery of refolded protein. In the cases where a high yielding recovery process has been developed for refolding of the aggregated protein, inclusion body formation provides a straight forward strategy for recombinant protein purification. The higher the amount of this partially folded protein that is converted into the bioactive form, the more therapeutic protein can be recovered with improved yield and at low cost from inclusion bodies of E. coli.

It is necessary to have a method for the cloning, expression and purification of antibodies and preferably without cost-intensive chromatographic steps as well as extensive steps. The antibody obtained by the cloning, expression and purification method according to the present invention is supposed to meet the criteria for purity & yield which are set forth by the admission authorities.

OBJECT OF THE INVENTION

The principal object of the present invention is to use novel cloning processes followed by novel protein expression and its purification procedures for rapid and efficient recovery of recombinant Ranibizumab.

It is an object of the present invention to provide novel cloning process of Ranibizumab which comprises transforming the host cell with:

i) the vector comprising nucleic acid sequence of SEQ ID No. 1 encoding for light chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 2 wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence, start codon and an inducible promoter system

ii) transforming another host cell with another plasmid vector comprising nucleic acid sequence of SEQ ID No. 3 encoding for heavy chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 4 wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence, start codon and an inducible promoter system.

Another object of the present invention is to provide novel protein expression method for the preparation of Ranibizumab which comprises steps of:

i) expressing light & heavy chain separately in two different expression host cells

ii) exporting protein to the periplasmic space of the cells with the help of a unique signal sequence

iii) partially pure light and heavy chain proteins are refolded together in-vitro

Yet another object of the present invention is to provide novel protein purification method of Ranibizumab having an amino acid sequence of SEQ. ID. No. 2 and 4, which comprises:

i) high cell density culturing of the host cells in a growth medium by maintaining specific culture conditions

ii) expression of the protein in the form of periplasmic inclusion bodies

iii) protein refolding of both chains together in-vitro

iv) purification of correctly folded protein

Yet another object of the present invention is to provide improved purification process of Ranibizumab which comprises:

i) in-vitro refolding of protein

ii) performing anion exchange chromatography to separate out closely related misfolded protein species

iii) performing cation exchange Chromatography followed by

iv) ultra filtration/Diafiltration

Further object of the present invention is to provide improved purification process of Ranibizumab which is capable to separate out even closely related misfolded protein species (>95% pure protein).

Yet another object of the invention is to provide a nucleic acid sequence of SEQ ID No. 1 which encodes for light chain of Ranibizumab wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence (SEQ ID No. 5).

Yet another object of the invention is to provide a nucleic acid sequence of SEQ ID No. 3 which encodes for heavy chain of Ranibizumab wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence (SEQ ID No. 6).

Yet another object of the invention is to provide use of unique signal sequence containing the amino acid sequence of SEQ ID No. 5 for production of anti-VEGF antibody.

Yet another object of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 6 for production of anti-VEGF antibody.

Yet another object of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 5 for production of light chain of Ranibizumab.

Yet another object of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 6 for production of heavy chain of Ranibizumab.

SUMMARY OF THE INVENTION

The principal aspect of the present invention is to use novel cloning processes followed by novel protein expression and its purification procedures for rapid and efficient recovery of recombinant Ranibizumab.

It is an aspect of the present invention to provide novel cloning process of Ranibizumab which comprises transforming the host cell with:

i) the vector comprising nucleic acid sequence of SEQ ID No. 1 encoding for light chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 2 wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence, start codon and an inducible promoter system

ii) transforming another host cell with another plasmid vector comprising nucleic acid sequence of SEQ ID No. 3 encoding for heavy chain of Ranibizumab having an amino acid sequence as shown

in SEQ ID No. 4 wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence, start codon and an inducible promoter system.

Another aspect of the present invention is to provide novel protein expression method for the preparation of Ranibizumab which comprises steps of:

i) expressing light & heavy chain separately in two different expression host cells
ii) exporting protein to the periplasmic space of the cells with the help of a unique signal sequence
iii) partially pure light and heavy chain proteins are refolded together in-vitro

Yet another aspect of the present invention is to provide novel protein purification method of Ranibizumab having an amino acid sequence of SEQ. ID. No. 2 and 4, which comprises:

i) high cell density culturing of the host cells in a growth medium by maintaining specific culture conditions
ii) expression of the protein in the form of periplasmic inclusion bodies
iii) protein refolding of both chains together in-vitro
iv) purification of correctly folded protein

Yet another aspect of the present invention is to provide improved purification process of Ranibizumab which comprises:

i) in-vitro refolding of protein
ii) performing anion exchange chromatography to separate out closely related misfolded protein species
iii) performing cation exchange Chromatography followed by
iv) ultra filtration/Diafiltration

Further aspect of the present invention is to provide improved purification process of Ranibizumab which is capable to separate out even closely related misfolded protein species (>95% pure protein).

Yet another aspect of the invention is to provide a nucleic acid sequence of SEQ ID No. 1 which encodes for light chain of Ranibizumab wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence (SEQ ID No. 5).

Yet another aspect of the invention is to provide a nucleic acid sequence of SEQ ID No. 3 which encodes for heavy chain of Ranibizumab wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence (SEQ ID No. 6).

Yet another aspect of the invention is to provide use of unique signal sequence containing the amino acid sequence of SEQ ID No. 5 for production of anti-VEGF antibody.

Yet another aspect of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 6 for production of anti-VEGF antibody.

Yet another aspect of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 5 for production of light chain of Ranibizumab.

Yet another aspect of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 6 for production of heavy chain of Ranibizumab.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 represents expression plasmid carrying Ranibizumab light chain gene construct
Fig. 2 represents expression plasmid carrying Ranibizumab heavy chain gene construct
Fig.3 represents characterization of recombinant protein expressed using above process
Fig.4a represents CD Spectra of properly folded Ranibizumab compared with Lucentis
Fig.4b represents fluorescence Spectra of properly folded Ranibizumab compared with Lucentis
Fig.5 represents cell based assay showing comparative potency of recombinant Ranibizumab compared with Lucentis

DETAILED DESCRIPTION OF THE INVENTION

Ranibizumab is an affinity maturated Fab fragment derived from bevacizumab. Ranibizumab has a higher affinity for VEGF and also is smaller in size, allowing it to better penetrate the retina, and thus treat the ocular neovascularization associated with AMD (Lien and Lowman, In: Chemajovsky, 2008, Therapeutic Antibodies. Handbook of Experimental Pharmacology 181, Springer-Verlag, Berlin Heidelberg 131-150). Ranibizumab was developed and is marketed by Genentech under the trade name Lucentis.

LUCENTIS is a sterile, colorless to pale yellow solution in a single-use glass vial. Ranibizumab, which

lacks an Fc region, has a molecular weight of approximately 48 kilodaltons and is produced by an E. coli expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.

Unless indicated otherwise, the term "VEGF-binding molecule" includes anti-VEGF antibodies, anti-VEGF antibody fragments, "anti-VEGF antibody-like molecules" and conjugates with any of these. Antibodies include, but are not limited to, monoclonal and chimerized monoclonal antibodies. The term "antibody" encompasses complete immunoglobulins, like monoclonal antibodies produced by recombinant expression in host cells, as well as VEGF-binding antibody fragments or "antibody-like molecules", including single-chain antibodies and linear antibodies, so-called "SMIPs" ("Small Modular Immunopharmaceuticals"), as e.g. described in WO02/056910. Anti-VEGF antibody-like molecules include immunoglobulin single variable domains, as defined herein. Other examples for antibody-like molecules are immunoglobulin super family antibodies (IgSF), or CDR-grafted molecules.

"VEGF-binding molecule" refers to both monovalent VEGF-binding molecules (i.e. molecules that bind to one epitope of VEGF) as well as to bi- or multivalent binding molecules (i.e. binding molecules that bind to more than one epitope, e.g. "biparatopic" molecules as defined hereinbelow).

Signal sequence refers to a sequence present on the N-terminal side of a secretory protein precursor but absent in the naturally-occurring mature protein, and a "signal peptide" refers to a peptide cleaved from such a protein precursor. In general, a signal sequence is cleaved by a protease (typically referred to as a signal peptidase) when secreted extracellularly. Although such signal peptides have constant, common features in their sequences among biological species, a signal peptide which exhibits a secretory function in a certain biological species does not necessarily exhibit a secretory function in another biological species.

Inclusion bodies are dense electron-refractile particles of aggregated protein found in both the cytoplasmic and periplasmic spaces of E. coli during high-level expression of heterologous protein. It is generally assumed that high level expression of non-native protein (higher than 2% of cellular protein) and highly hydrophobic protein is more prone to lead to accumulation as inclusion bodies in E. coli. In the case of proteins having disulfide bonds, formation of protein aggregates as inclusion bodies is anticipated since the reducing environment of bacterial cytosol inhibits the formation of disulfide bonds.

Inclusion bodies have higher density (~1.3 mg ml–1) than many of the cellular components, and thus can

be easily separated by high-speed centrifugation after cell disruption. Expression of recombinant proteins as inclusion bodies in bacteria is one of the most efficient ways to produce cloned proteins, as long as the inclusion body protein can be successfully refolded. Aggregation is the leading cause of decreased refolding yields.

Protein refolding refers to the process by which a protein structure assumes its functional shape or conformation. It is the physical process by which a polypeptide folds into its characteristic and functional three-dimensional structure from random coil. Each protein exists as an unfolded polypeptide or random coil when translated from a sequence of mRNA to a linear chain of amino acids. This polypeptide lacks any stable (long-lasting) three-dimensional structure. Amino acids interact with each other to produce a well-defined three-dimensional structure, the folded protein, known as the native state. The resulting three-dimensional structure is determined by the amino acid sequence. Insoluble, inactive inclusion bodies are frequently formed upon recombinant protein production in transformed microorganisms. These inclusion bodies, which contain the recombinant protein in an highly enriched form, can be isolated by solid/liquid separation. After solubilization, native proteins can be generated from the inactive material by using in vitro folding techniques.

The present invention provides new refolding procedure for Ranibizumab comprising efficient in vitro reconstitution of complex hydrophobic, multidomain, oligomeric, or highly disulfide-bonded proteins. These protocols take into account process parameters such as protein concentration, catalysis of disuffide bond formation, temperature, pH, and ionic strength, as well as specific solvent ingredients that reduce unproductive side reactions.

Clone refers to a DNA sequence, such as a gene, that is transferred from one organism to another and replicated by genetic engineering techniques. The present invention comprises genes of heavy and light chain of Ranibizumab with desired modifications for cloning on both the ends. The gene sequences are optimized for better protein expression in E. coli. The synthetic constructs are made so as to have a bacterial leader signal sequence at the N-terminal followed by sequences of gene of interest and two translation stop codons in the end. These signal sequences are selected on the basis of their ability so that they can transport maximum protein in the periplasm of the cell. The signal sequence chosen was taken from a natural bacterial gene.

The main embodiment of the present invention is to use novel cloning processes followed by novel protein expression and its purification procedures for rapid and efficient recovery of recombinant

Ranibizumab.

It is yet another embodiment of the present invention to provide novel cloning process of Ranibizumab which comprises transforming the host cell with:

i) the vector comprising nucleic acid sequence of SEQ ID No. 1 encoding for light chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 2 wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence, start codon and an inducible promoter system

ii) transforming another host cell with another plasmid vector comprising nucleic acid sequence of SEQ ID No. 3 encoding for heavy chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 4 wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence, start codon and an inducible promoter system.

Another embodiment of the present invention is to provide novel protein expression method for the preparation of Ranibizumab which comprises steps of:

i) expressing light & heavy chain separately in two different expression host cells

ii) exporting protein to the periplasmic space of the cells with the help of a unique signal sequence

iii) partially pure light and heavy chain proteins are refolded together in-vitro.

Another embodiment of the present invention is to provide novel protein purification method of Ranibizumab having an amino acid sequence of SEQ. ID. No. 2 and 4, which comprises:

i) high cell density culturing of the host cells in a growth medium by maintaining specific culture conditions

ii) expression of the protein in the form of periplasmic inclusion bodies

iii) protein refolding of both chains together in-vitro

iv) purification of correctly folded protein

Yet another embodiment of the present invention is to provide improved purification process of Ranibizumab which comprises:

i) in-vitro refolding of protein

ii) performing anion exchange chromatography to separate out closely related misfolded protein species

iii) performing cation exchange Chromatography followed by

iv) ultra filtration/Diafiltration

Yet another embodiment of the present invention is to provide improved purification process of

Ranibizumab which is capable to separate out even closely related misfolded protein species (>95% pure protein).

Yet another embodiment of the invention is to provide a nucleic acid sequence of SEQ ID No. 1 which encodes for light chain of Ranibizumab wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence (SEQ ID No. 5).

Yet another embodiment of the invention is to provide a nucleic acid sequence of SEQ ID No. 3 which encodes for heavy chain of Ranibizumab wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence (SEQ ID No. 6).

Yet another embodiment of the invention is to provide use of unique signal sequence containing the amino acid sequence of SEQ ID No. 5 for production of anti-VEGF antibody.

Yet another embodiment of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 6 for production of anti-VEGF antibody.

Yet another embodiment of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 5 for production of light chain of Ranibizumab.

Yet another embodiment of the invention is to provide use of a unique signal sequence containing the amino acid sequence of SEQ ID No. 6 for production of heavy chain of Ranibizumab.

Light Chain Nucleotide Sequence **(SEQ ID NO: 1)**

5′GAGCT*CCATGG*AGTTTTTCAAAAAGACGGCACTTGCCGCACTGGTTATGGGTTTTAGTGGTGCAGCATTG
GCC*GATATC*CAGCTGACCCAGAGCCCGAGCAGCCTGAGCGCAAGCGTTGGTGATCGTGTTACCATTACCTG
TAGCGCAAGCCAGGATATTAGCAATTATCTGAATTGGTATCAGCAGAAACCGGGTAAAGCACCGAAAGTTC
TGATTTATTTTACCAGCAGCCTGCATAGCGGTGTTCCGAGCCGTTTTAGCGGTAGCGGTAGTGGCACCGAT
TTTACCCTGACCATTAGCAGCCTGCAGCCGGAAGATTTTGCAACCTATTATTGTCAGCAGTATAGCACCGT
TCCGTGGACCTTTGGTCAGGGCACCAAAGTTGAAATTAAACGTACCGTTGCAGCACCGAGCGTTTTTATTT
TTCCGCCTAGTGATGAACAGCTGAAAAGCGGCACCGCAAGCGTTGTTTGTCTGCTGAATAATTTTTATCCG
CGTGAAGCAAAAGTGCAGTGGAAAGTTGATAATGCACTGCAGAGCGGTAATAGCCAAGAAAGCGTTACCGA
ACAGGATAGCAAAGATAGCACCTATAGCCTGAGCAGCACCCTGACCCTGAGCAAAGCAGATTATGAAAAAC
ACAAAGTGTATGCCTGCGAAGTTACCCATCAGGGTCTGAGCAGTCCGGTTACCAAAAGTTTTAATCGTGGC
GAATGCTAATAG*AAGCTT*GGTACC 3′

Light Chain Amino acid Sequence (SEQ ID NO: 2)

DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPK
VLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYS
TVPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLN
NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Heavy Chain Nucleotide Sequence (SEQ ID NO: 3)

5′GAGCT*CATATG*AAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGC
GATGGCCGAAGTTCAGCTGGTTGAAAGCGGTGGTGGTCTGGTTCAGCCTGGTGGTAGCCTGCGTCTGAG
CTGTGCAGCAAGCGGTTATGATTTTACCCATTATGGTATGAATTGGGTTCGTCAGGCACCGGGTAAAGG
TCTGGAATGGGTTGGTTGGATTAATACCTATACCGGTGAACCGACCTATGCAGCAGATTTTAAACGTCG
TTTTACCTTTAGCCTGGATACCAGCAAAAGCACCGCATATCTGCAGATGAATAGCCTGCGTGCAGAAGA
TACCGCAGTTTATTATTGTGCCAAATATCCGTATTACTATGGCACCAGCCACTGGTATTTCGATGTTTG
GGGTCAGGGCACCCTGGTTACCGTTAGCAGCGCAAGCACCAAAGGTCCGAGCGTTTTTCCGCTGGCACC
GAGCAGCAAAAGTACCAGCGGTGGCACAGCAGCACTGGGTTGTCTGGTTAAAGATTATTTTCCGGAACC
GGTTACCGTGAGCTGGAATAGCGGTGCACTGACCAGCGGTGTTCATACCTTTCCGGCAGTTCTGCAGAG
CAGCGGTCTGTATAGCCTGAGCAGCGTTGTTACCGTTCCGAGCAGCAGCCTGGGCACCCAGACCTATAT
TTGTAATGTTAATCATAAACCGAGCAATACCAAAGTGGATAAAAAAGTTGAGCCGAAAAGCTGCGATAA
AACCCATCTGTAATAG*GGTACC* 3′

Heavy Chain Amino acid Sequence (SEQ ID NO: 4)

EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPG
KGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSL
RAEDTAVYYCAKYPYYYGTSHWYFDVWGQGTLVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH
TFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK
KVEPKSCDKTHL

The amino acid sequence of the unique signal sequence for light chain of Ranibizumab is:

MEFFKKTALAALVMGFSGAALA (SEQ ID No: 5)

The amino acid sequence of the unique signal sequence for heavy chain of Ranibizumab is:

MKYLLPTAAAGLLLLAAQPAMA (SEQ ID No: 6)

The invention will now be further described by the following examples, which are illustrative rather than limiting.

Example 1

Generation of pCDL18-LC vector for light chain of Ranibizumab

Bacterial expression vector for light chain of Ranibizumab was generated by cloning light chain of Ranibizumab along with a signal sequence (SEQ ID No: 1) at the 5' end into the NcoI/HindIII site in multiple cloning site (MCS) of pCDL18-LC vector.

Key components of synthetic gene cassette and schematic design are as given below.



Generation of pCDL18-HC vector for heavy chain of Ranibizumab

Bacterial expression vector for heavy chain of Ranibizumab was generated by cloning heavy chain of Ranibizumab along with a signal sequence (SEQ ID No: 3) at the 5' end into the NdeI/XhoI site in pCDL18-HC vector.

Key components of synthetic gene cassette and schematic design are as given below.



Example 2

Transformation of light chain gene in BL21 (DE3)

pSR04 vector carrying expression construct for light chain gene (pCDL18-LC) was transformed in BL21 (DE3) and recombinant clones were selected. The transformants were plated on LB agar plates containing kanamycin (30 µg/ml) for selection. Protein expression analysis was also performed after inducing the cells with 1 mM IPTG for 4 h in a shake flask. Whole cell lysate and extracellular protein samples were

analyzed for clone selection.

Transformation of heavy chain gene in BL21 (DE3)

pSR02 vector carrying expression construct for heavy chain gene (pCDL18-HC) was transformed in BL21 (DE3) and recombinant clones were selected. The transformants were plated on LB agar plates containing ampicillin for selection. Protein expression analysis was also performed after inducing the cells with 1 mM IPTG for 4 h in a shake flask. Whole cell lysate and extracellular protein samples were analyzed for clone selection.

Example 3

General expression of light & heavy chain separately in two different expression host cells in E.*coli*

To maximize the desired protein expression the light and heavy chains of Ranibizumab were cloned into two separate vector systems and transformed individually in two different E. coli cells. The protein expression is derived from T7 promoter system. Both these constructs are carried in by these high copy number plasmids and capable of expressing protein in a tightly regulated manner.

The overexpressed recombinant proteins are purified and characterized. The first 10 residues at the N-terminal were confirmed to be DIQLTQSPSS for light chain and EVQLVESGGG for the heavy chain, which is the authentic start sequence of both the chains. A clear and unambiguous signal was obtained for all the 10 residues.

Example 4

Generation of targeted protein in form of periplasm inclusion bodies

The recombinant E.coli cells were cultivated in the shake flasks (seed flasks) for inoculum preparation and for production, the inoculum obtained from seed flask was transferred to production fermenter and cultured for 25 h in fed batch mode. During fermentation, the cells were provided with air and oxygen by means of sparging. The growth of the cells was maintained by controlled addition of feed (Nutrient supplements) in pH stat mode, the pH was maintained by supplying glucose and nitrogen in feed to reduce the pH of the batch. Base (NaOH) was used to increase the pH of the batch as needed. Cells were induced with IPTG at 20th hour of batch age and the fermentation is carried out for another 5 hours. The targeted protein is produced in the form of periplasmic IBs.

For light chain the cell culture density (OD600) at harvest was ~50 and biomass obtained from that was around 100g/L, which yielded around 25g IBs/L.

Similarly for heavy chain the cell culture density (OD600) at harvest was ~100 and biomass obtained was around 155g/L which yielded around 33g IBs/L.

Example 5

Refolding of light and heavy chain together

25 g of LC and HC each were solubilized separately in 500 mL of solubilization buffer containing 6 M GuHCl and pooled in 1:1 ratio. Pooled SIB was reduced with 4mM of DTT for 1 h. Reduced LC and HC pool was subjected to oxidation with 10 mM cystine and incubated for 3 h. Reduced and oxidized SIB were diluted 25 times in the refolding buffer (100 mM Tris, 0.6 M Arginine, 5 % Sorbitol, 2 mM EDTA, pH 9.0) by slow addition. The 0.6 mM of cystine and 0.75 mM of cysteine was added to the refolding mixture and reaction was incubated at (2-8)°C for ~ 5 days. Refolding output was concentrated using 10 kDa membrane and diafiltered against 50 mM Tris buffer, pH 9.0.

Yield and purity after refolding: Properly folded Ranibizumab yield after refolding was around 18% of the total protein.

Example 6

Purification for properly folded protein

Refolding output was concentrated using 10 kDa membrane and diafiltered against 50 mM Tris buffer pH 9.0. Diafiltration output was loaded on Q Sepharose FF resin in binding mode and protein was eluted out by reducing the pH to 6.7 in linear gradient in 10 CV from 0 % B to 100 % B. Q Sepharose Output was loaded on SP Sepharose HP resin at pH 5.0. Protein was eluted out by increasing salt concentration as follows 20 % step followed by 20 % to 50 % gradient and finally a 100 % step gradient. Pooled fractions were concentrated and diafiltered against the formulation buffer using a 10 kDa membrane.

Yield of final product after purification: The overall protein purification process recovery was around 9% with a purity level of >99%.

WE CLAIM,

1. A polynucleotide sequence of SEQ ID No. 1 encoding for a unique signal sequence of SEQ ID No. 5 and the light chain of Ranibizumab of SEQ ID No. 2 wherein N-terminal of the said SEQ ID No. 2 is operably linked to a unique signal sequence of SEQ ID No. 5.

2. A polynucleotide sequence of SEQ ID No. 3 encoding for a unique signal sequence of SEQ ID No. 6 and the heavy chain of Ranibizumab of SEQ ID No. 4 wherein N-terminal of the said SEQ ID No. 4 is operably linked to a unique signal sequence of SEQ ID No. 6.

3. Use of a unique signal sequence comprising the amino acid sequence of SEQ ID No. 5 for production of anti-VEGF antibody.

4. Use of a unique signal sequence comprising the amino acid sequence of SEQ ID No. 6 for production of anti-VEGF antibody.

5. Use of a unique signal sequence comprising the amino acid sequence of SEQ ID No. 5 for production of light chain of Ranibizumab.

6. Use of a unique signal sequence comprising the amino acid sequence of SEQ ID No. 6 for production of heavy chain of Ranibizumab.

7. A recombinant vector comprising the nucleic acid sequence of SEQ ID No. 1 encoding for the light chain of Ranibizumab as claimed in claim 1.

8. A recombinant vector comprising the nucleic acid sequence of SEQ ID No. 3 encoding for the heavy chain of Ranibizumab as claimed in claim 2.

9. A host cell comprising the recombinant vector as claimed in claim 7 containing the nucleotide sequence encoding for the light chain of Ranibizumab as claimed in claim 1.

10. A host cell comprising the recombinant vector as claimed in claim 8 containing the nucleotide

10. A host cell comprising the recombinant vector as claimed in claim 8 containing the nucleotide sequence encoding for the heavy chain of Ranibizumab as claimed in claim 2.

11. A novel cloning process for the preparation of Ranibizumab comprising transforming the host cell with:
    a) the vector comprising nucleic acid sequence of SEQ ID No. 1 encoding for light chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 2 wherein N-terminal of the said SEQ ID No. 1 is operably linked to a unique signal sequence, start codon and an inducible promoter system
    b) transforming another host cell with another plasmid vector comprising nucleic acid sequence of SEQ ID No. 3 encoding for heavy chain of Ranibizumab having an amino acid sequence as shown in SEQ ID No. 4 wherein N-terminal of the said SEQ ID No. 3 is operably linked to a unique signal sequence, start codon and an inducible promoter system.

12. A process for the production of light chain of Ranibizumab as claimed in claim 11 wherein the process comprises
    a) transforming the host cell with the vector as claimed in claim 7
    b) culturing the high cell density host cell in a growth medium by maintaining specific culture composition
    c) expressing the light chain separately in a host cell as claimed in claim 9
    d) exporting light chain protein to the periplasmic space of the cells with the help of a unique signal sequence.

13. A process for the production of heavy chain of Ranibizumab as claimed in claim 11 wherein the process comprises:
    a) transforming the host cell with the vector as claimed in claim 8
    b) culturing the high cell density host cell in a growth medium by maintaining specific culture composition
    c) expressing the heavy chain separately in a host cell as claimed in claim 10
    d) exporting heavy chain protein to the periplasmic space of the cells with the help of a unique signal sequence.

14. A novel expression process for the production of light chain and heavy chain of Ranibizumab having

amino acid sequences of SEQ ID No. 2 and SEQ ID No. 4 respectivy wherein the process comprises:

a) Separately culturing the host cells carrying the genes for the light chain and heavy chain of Ranibizumab in a growth medium by maintaining specific culture composition
b) Expression of the light and heavy chain in the form of periplasmic inclusion bodies
c) Solubilization of the inclusion bodies
d) In-vitro refolding of the solubilized light chain and heavy chain of Ranibizumab and clarification.

15. A novel process comprising the reduction, oxidation and subsequent in-vitro refolding of the solubilized inclusion bodies to recover biologically active Ranibizumab.

16. A process for the purification of Ranibizumab obtained by the process as claimed in claim 15, wherein the purification process comprises the following steps:

a) Ultrafltration & diafiltration - I
b) Anion exchange chromatography
c) Cation exchange chromatography
d) Ultrafltration & diafiltration - II
e) 0.2 µm filtration.

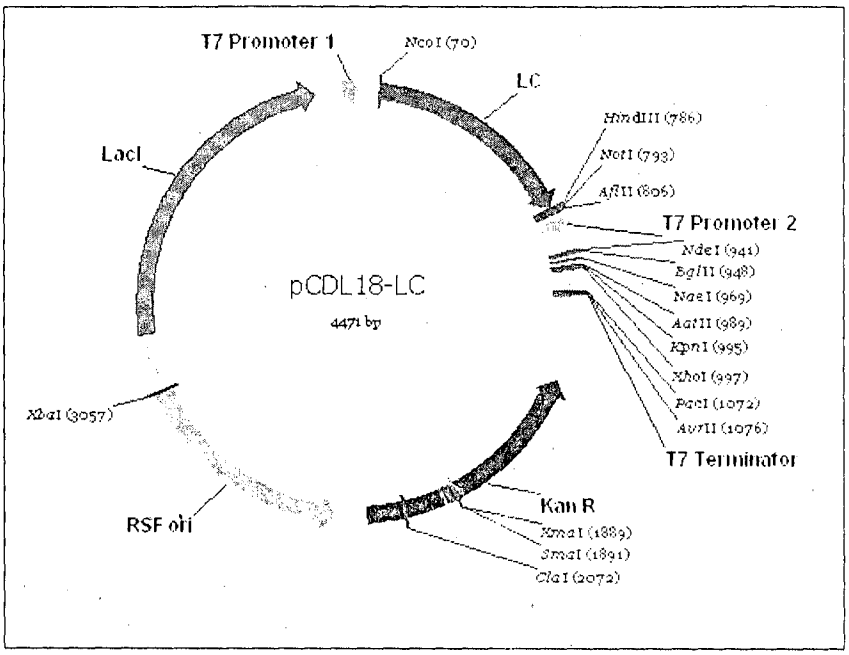


Figure – 1: pCDL18-LC vector carrying Ranibizumab Light Chain gene

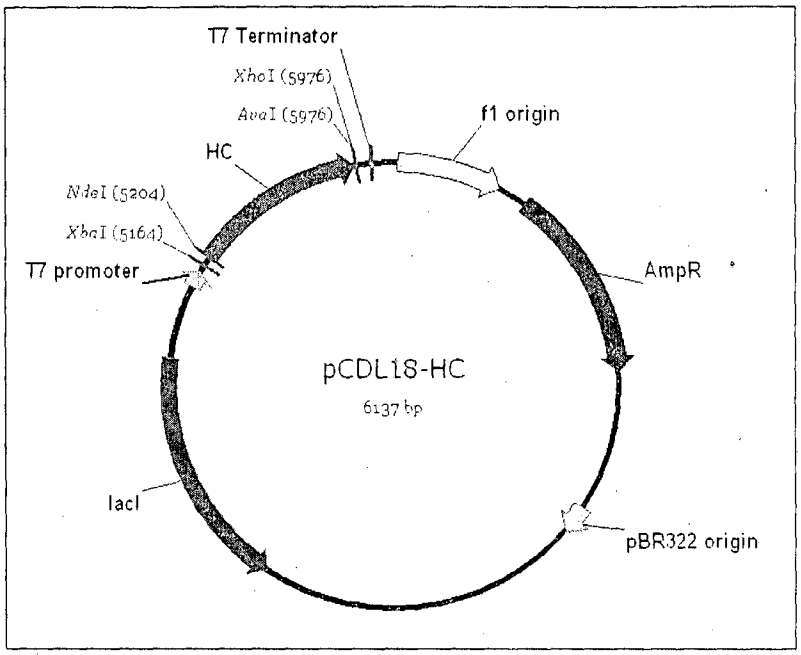
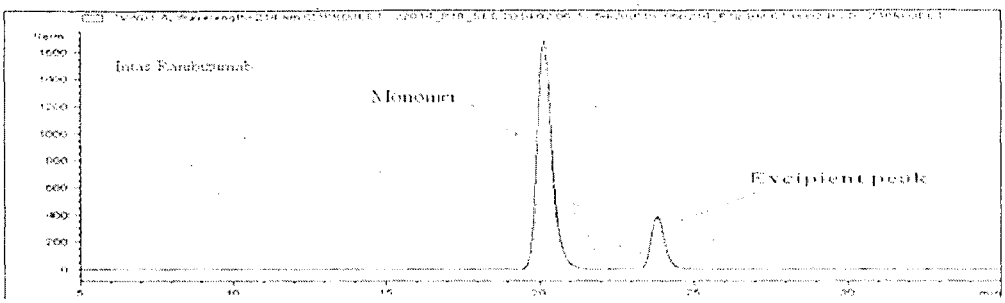
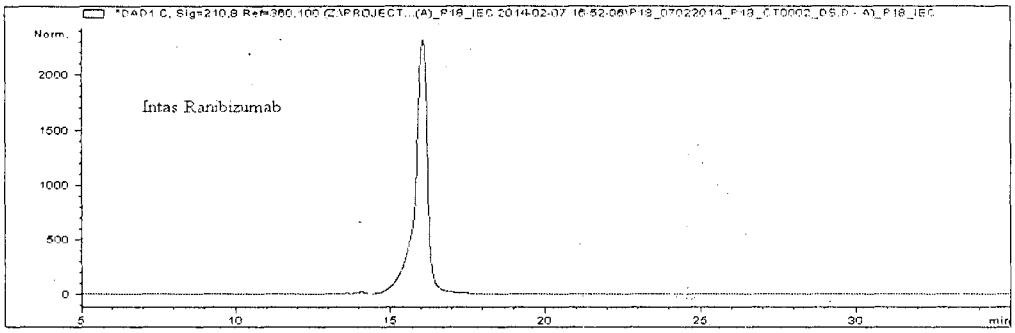


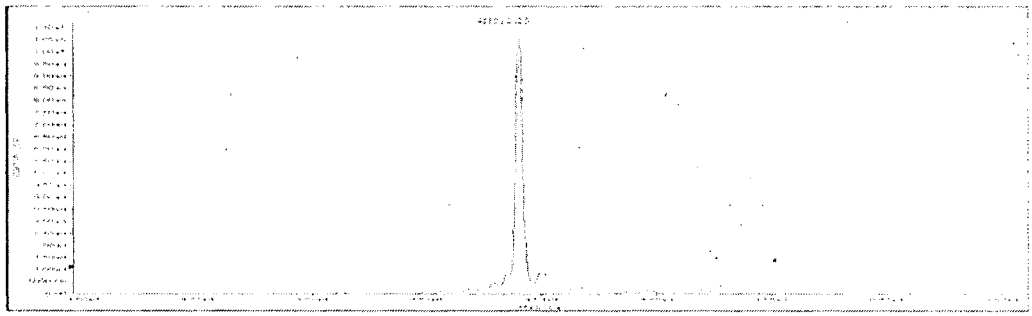
Figure – 2: pCDL18-HC vector carrying Ranibizumab Heavy Chain gene



SEC-HPLC profile of Intas Ranibizumab (99.9 % purity)



CEX-HPLC profile of Intas Ranibizumab (98.7 % purity)



Mass spectra profile of Intas Ranibizumab (48.38kDa)

Figure – 3: Characterization of recombinant protein expressed using above process

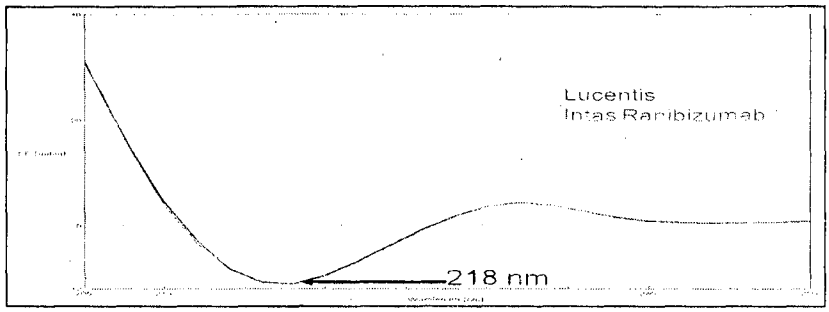


Figure – 4a: CD Spectra of properly folded Ranibizumab compared with Lucentis

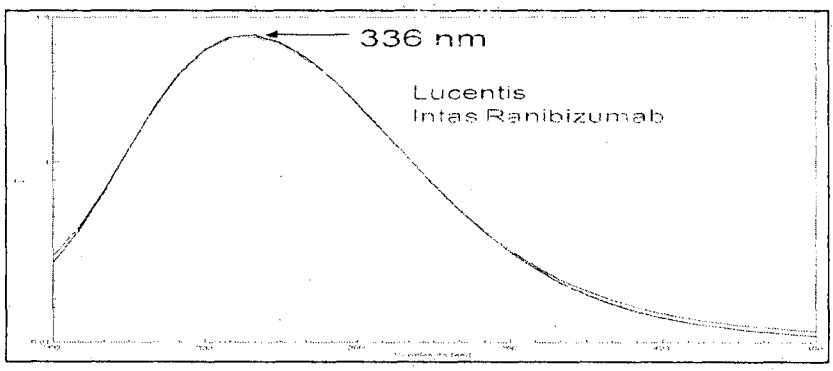


Figure – 4b: Fluorescence Spectra of properly folded Ranibizumab compared with Lucentis

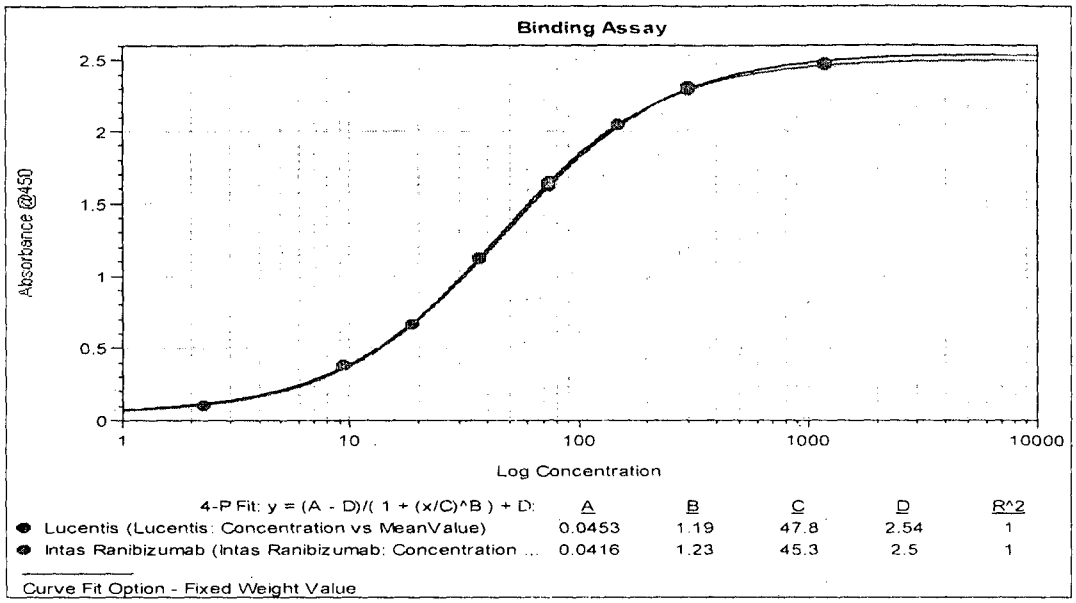


Figure – 5: Cell based assay showing comparative potency of recombinant Ranibizumab compared with Lucentis