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PERIPLASMIC EXPRESSION, RECOVERY AND QUANTIFICATION OF RECOMBINANT HUMAN INTERFERON-A2B IN FERMENTATION BY *ESCHERICHIA COLI*

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By

R. NAGASUNDARA RAMANAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

Dedicated to my beloved mother, family, friends and well wishers for their love, interest and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

Periplasmic Expression, Recovery and Quantification of Recombinant Human Interferon-A2b in Fermentation by *Escherichia coli*

By

R. NAGASUNDARA RAMANAN

October 2009

Chairman: Arbakariya B. Ariff, PhD

Faculty: Institute of Bioscience

Human interferon-α2b (IFN-α2b) is one of the biopharmaceuticals used to cure diseases such as hairy cell leukemia, malignant melanoma, and chronic hepatitis (B and C). Several areas related to the industrial problems, in the development of soluble IFN-α2b from recombinant *Escherichia coli* were explored in this study, which include enhancement of expression in periplasm, cell disruption techniques, quantification method and purification.

The use of pET 26b(+) plasmid enhanced the periplasmic expression of IFN-α2b (300 ng/mL) by about 3000 times in *E. coli* RG 2(DE3) as compared to that obtained in the previous recombinant strain (0.1 ng /mL) using pFLAG-ATS plasmid. Difference in the expression level was attributed to the difference in the promoters and the signal sequences. *In silico* analysis suggested that the enhancement was mainly due to the difference in the translation initiation caused by mRNA secondary structure of the plasmid.

The disruption of *E. coli* cells were investigated using glass bead shaking and homogenizer for small and large scale purpose, respectively. The optimum conditions for glass bead shaking were 30 min shaking at 300 rpm with 1.5 g/mL of glass beads (0.5 mm diameter). This technique was particularly useful for handling many samples at one time. The operating pressure range in a homogenizer was classified as low, transition and high pressures based on the characteristics of cell disintegrates. At low pressures, the protein release was mainly due to point break, which lead to high selectivity of IFN-α2b release. At higher pressures, the maximum release of total protein and IFN-α2b with a drastic reduction in cell size was observed after the first pass. Statistical optimization was used for osmotic shock process to release IFN-α2b at high concentration, with less process waste. Optimal process was achieved at cell concentration of 0.05 g/mL in hypertonic and 0.2 g/mL in hypotonic solutions.

A rapid immunoassay method for quantification of IFN-α2b was developed using surface plasmon resonance technique. Anti-interferon monoclonal antibody (anti-IFN) was immobilized onto the CM5 chip using an amine coupling method. The perfect linearity was observed between 10 and 200 ng/mL. The anti-IFN chip was found to be useful for more than 1000 cycles and could also be used in continuous running environment.

The efficacy of two activation methods using N-Hydroxysuccinimide in organic solvent (M I) and aqueous solution (M II) was assessed on CM Sepharose FF beads by immobilizing BSA onto it at various pH and ionic strengths. M I activation gave better immobilization efficiency than M II. Similar binding capacity was obtained

with beads immobilized at pH 5 and 8 using anti-IFN; and with crude IFN-α2b as ligand and ligate.

Knowledge gained from the molecular work gave better understanding of the expression pathway for future improvement of periplasmic IFN-α2b production by *E. coli*. Information and data obtained from this study were very useful for the development of efficient downstream and purification methods of IFN-α2b from *E. coli* fermentation at reduced cost, as well as simple and cheap quantification method for quality control and process monitoring.

Abstrak tesis yang dikemukan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

Pengekspresan Periplasmik, Pemulihan dan Pengiraan Rekombinan Interferon Manusia-A2b di dalam Fermentasi oleh *Escherichia coli*

By

R. NAGASUNDARA RAMANAN

Oktober 2009

Pengerusi: Arbakariya B. Ariff, PhD

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Interferon manusia-α2b (IFN-α2b) adalah satu daripada produk biofarmaseutikal yang digunakan untuk merawat pelbagai penyakit seperti leukemia sel berumbai, kanser kulit malignan, dan hepatitis B dan C yang kronik. Beberapa masalah berkaitan industri dalam penghasilan IFN-α2b dalam bentuk larut di dalam kawasan periplasmik *Escherichia coli* telah dikaji di dalam projek ini, termasuklah meningkatkan pengekspresan di dalam periplasmik, kaedah pemecahan sel, pengiraan dan proses penulenan.

Pemilihan pET 26b(+) sebagai plasmid meningkatkan pengekpresan IFN-α2b sebanyak 3000 kali ganda iaitu 300 ng/mL di dalam *E. coli* RG 2(DE3) berbanding 0.1 ng/mL yang diperolehi oleh strain rekombinan yang menggunakan plasmid pFLAG-ATS sebelum ini. Nilai pengekpresan yang berbeza ini telah disebabkan oleh perbezaan di antara promoter dan jujukan penanda. Hasil dari analisa *in silico,* perbezaan semasa permulaan penterjemahan yang menyebabkan peningkatan pengekspresan IFN-α2b adalah disebabkan oleh perbezaan struktur sekunder mRNA plasmid.

Pretasi pelbagai teknik, seperti goncangan butiran kaca, penghomogen dan kejutan osmotic, untuk pemecahan sel *E. coli* bagi melepaskan IFN-α2b daripada periplasmik dalam skala kecil dan besar juga telah dikaji. Keadaan yang paling optimum bagi teknik goncangan butiran kaca adalah menggunakan 1.5 g/mL butiran kaca (berdiameter 0.5mm) pada kelajuan 300 rpm selama 30 minit. Teknik ini adalah sangat berguna dalam skala kecil bagi mengendalikan sampel yang banyak pada masa yang sama. Kitaran bagi operasi tekanan penghomogen boleh dikelaskan kepada tekanan rendah, peralihan dan tinggi berdasarkan sifat-sifat pemecahan sel. Pada tekanan rendah, kebanyakan protein yang dilepaskan adalah disebabkan oleh pemecahan tunjuk yang menghasilkan pelepasan IFN-α2b dengan pemilihan yang tinggi. Pada tekanan tinggi, pembebasan yang maksima bagi kesemua protein dan IFN-α2b dengan pengurangan yang ketara dalam saiz sel adalah dilihat selepas laluan yang pertama. Keputusan yang optimum bagi teknik ini adalah sama dengan keputusan yang diperolehi daripada teknik ultrasonikasi dan gegaran butiran kaca. Purata kos dan masa untuk pemprosesan juga dinilaikan bagi semua tekanan. Pengoptimumam menggunakan kaedah statistik telah digunakan untuk proses kejutan osmotik untuk melepaskan IFN-α2b pada kepekatan yang tinggi, dengan sisa proses yang kurang. Daripada proses ini sebanyak 0.05 g/mL sel di dalam larutan hipertonik dan 0.2 g/mL di dalam larutan hipotonik adalah yang paling optimum.

Kaedah immunoesei untuk menentukan kuantiti IFN-α2b telah dihasilkan menggunakan teknik ”surface plasmon resonance” (SPR). Antibodi monoklonal anti interferon (anti-IFN) adalah dipegun ke atas cip CM5 menggunakan kaedah pengikatan amina. Keselarian sempurna telah diperhatikan di antara 10 dan 200

ng/mL. Cip anti IFN ini dapat bertahan untuk lebih daripada 1000 kitaran dan juga penggunaan secara berterusan.

Ketepatan dua kaedah pengaktifan menggunakan N-Hydroxysuccinimide dalam pelarut organik (M I) dan larutan (M II) telah dikaji pada manik CM Sepharose FF dengan menahan BSA ke atasnya pada pH dan kekuatan ion yang berbeza. Pengaktifan MI telah menghasilkan ketepatan penahanan yang lebih baik berbanding M II. Manik pegun pada pH 5 dan 8 menggunakan anti-IFN dan standard IFN-α2b sebagai ligan dan ligat menunjukkan keupayaan pengikatan yang serupa.

Pengetahuan yang diperolehi daripada kajian sel biomolekul memberikan kefahaman berkaitan pengekspresan untuk peningkatan hasil IFN-α2b dalam periplasmik daripada *E. coli*. Maklumat dan data yang diperolehi daripada kajian ini adalah berguna dalam pembanguanan pemprosesan hiliran dan kaedah yang efektif untuk menulenkan IFN-α2b daripada proses fermentasi *E. coli* dengan kos yang rendah, dan juga cara penentuan kuantiti yang mudah dan murah untuk pengawalan mutu dan pemantauan proses.

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I certify that a Thesis Examination Committee has met on 27.10.2009 to conduct the final examination of R. Nagasundara Ramanan on his thesis entitled "Periplasmic Expression, Recovery and Quantification of Recombinant Human Interferon-A2b in Fermentation by *Escherichia coli*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 14 January 2010

DECLARATION

I declare that the thesis is my original work except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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R. NAGASUNDARA RAMANAN

Date:

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