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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.1 7(H))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(Hi))*

— *of inventorship (Rule 4.1 7(iv))*

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(54) Title: PROCESS FOR THE PURIFICATION OF RECOMBINANT HUMAN IL-1 1

(57) Abstract: The invention relates to process for purification of recombinant human IL-1 1 from microbial cells. The method involves purification using hydrophobic interaction chromatography and ion exchange chromatography. The method further comprises use one or more fusion tags which provides solubility to the protein and also simplifies the purification of the target protein.

PROCESS FOR THE PURIFICATION OF RECOMBINANT HUMAN IL-11

Field of the invention

The invention relates to process for purification of recombinant human IL-1 1 from a soluble IL-1 1 fusion protein expressed in bacterial system. The method involves purification of the soluble fusion protein, digestion to separate the IL-1 1 from the fusion tag followed by hydrophobic interaction chromatography and ion exchange chromatography.

Background of the invention

The development of techniques and methods for protein purification has been an essential prerequisite for many of the advancements made in biotechnology. Protein purification varies from simple one step precipitation procedures to large scale validated production processes. The key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required. Most commonly used purification methods include use of affinity tags, fusion tags, metal binding, immunoaffinity chromatography, ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography and HPLC.

Recombinant DNA developments over the past decade have revolutionized the production of proteins in large quantities. While production of heterologous proteins in bacterial hosts has been implemented successfully in the biotechnology industry, there are numerous instances where bacterial expression systems have given less than satisfactory results. Often, over expression leads to the production of inclusion bodies, that are insoluble aggregates of misfolded proteins. For example, when high-expression levels are achieved, recombinant proteins are frequently expressed in ***Escherichia coli*** as insoluble protein aggregates termed "inclusion bodies" that have been the subject of many protein folding studies. The conditions for refolding the denatured protein must be optimized for each specific protein, and the renaturation yield may be low even in an optimized system. Thus it is often desirable to maximize the expression of the protein in a completely soluble form.

US 5215895 relates to a novel cytokine that stimulates the function of cells of the immune and hematopoietic systems, and to processes for obtaining the factor and producing it by recombinant genetic engineering techniques. It describes expression of IL-l l in bacterial cells where the purification involves four ion exchange steps and cleavage of the fusion protein by hydroxylamine.

WO9516044A2 relates to proteins and peptide(s) fused to thioredoxin or thioredoxin-like molecules - useful for production of large amounts of heterologous proteins. The fusion molecule is modified to introduce one or more metal-binding/chelating amino-acid residues to aid in purification. Expression of this fusion molecule under the control of a regulatory sequence capable of directing its expression in a desired host cell produces high levels of stable and soluble fusion protein.

EP **1598364A1** discloses novel polynucleotide encoding fusion interleukin (IL)-l l receptor and **IL-1 1** polypeptide.

US 20090036652A1, WO 06126102A2 and **US 5646016** disclose method for purification of fusion using different purification protocols.

US 20070 141 662A1 discloses a method for recombinantly producing a peptide comprising expressing the peptide as a fusion protein and applying the protease to cleave the fusion protein.

WO 9521 197A1, US 20070 14 1662A1, EP **888384B1, US 7585943,** and **US 7442371** relates to fusion products prepared by recombinant **DNA** procedures.

Summary of the invention

In one general aspect the invention is related to a method for purification of recombinant human Interleukin- **11** from bacterial cells, the process comprising the steps of:

(a) purifying the fusion protein using hydrophobic interaction chromatography,

(b) cleaving the fusion protein with enterokinase, and

(c) purifying **IL-1 1** by ion exchange chromatography,

wherein the purification steps may be in any order.

In another aspect of the invention the hydrophobic interaction chromatography is performed using resins selected from butyl sepharose, phenyl sepharose or octyl sepharose. In another aspect of the invention the Ion exchange chromatography is selected from cation exchange or anion exchange or both in either order.

In yet another aspect of the invention the method for purification of recombinant human Interleukin 11 comprises use of one or more fusion tags selected from SD tag, GM tag, T7 tag, GST tag, His tag, Trx Tag or MBP tag. The SD tag used in the invention having sequence Id 1, comprises of 49 times repeated, Serine Aspartate residues. The fusion tag (having a pi of 4.0) has a total of 107 amino acids.

The details of one or more embodiments of the inventions are set forth in the description below. Other features, objects and advantages of the inventions will be apparent from the description and claims.

Brief description of the accompanying drawings:

Figure 1: SDS PAGE separation of Interleukin 11.

DESCRIPTION OF SEQUENCE ID

SEQ ID 1: Amino acid sequence of SD tag

**MSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSD
SDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSGSDDDDK.**

SEQ ID 2: Nucleotide sequence of SD tag

**ATGAGCGATTCCGATTCAGACTCGGACTCGGATTCCGATTCCGACAGTGATTCAGATTCTGACT
CAGATTCCGATTCTGATTCTGATTCGGATTCCGACTCCGATAGCGACTCAGATAGTGACTCTGA
CTCGGACAGCGATTCTGATAGCGACTCTGATTCCGATAGCGATAGCGATTCAGATAGCGATTC
TGACTCGGATTCTGATTCCGATTCTGACTCTGACAGCGATTCCGATAGCGACAGCGACTCTGAT
AGTGATTCAGACTCTGATTCTGATAGTGATAGCGATTCGGATAGTGGATCCGATGATGATGAT
AAA.**

SEQ ID 3: Amino acid sequence of IL 11

GPPPGPPRAELDSTVLLTRSLLADTRQLAAQLRDKFPADGDHNLDSLPTLAMSAGALGALQLPGVL TRLRADLLSYLRHVQWLRRAGGSSLKTLEPELGTLQARLDRLLRRLQLLMSRLALPQPPPDPPAPP LAPPSSAWGGIRAAHAILGGLHLTLDWAVRGLLLLKTRL.

SEQ ID 4: Nucleotide sequence of IL 11

GGTCCACCACCTGGACCACCTCGGGCCGAGCTGGACAGCACCGTGCTCCTGACCCGCTCTCTC CTGGCGGACACGCGGCAGCTGGCTGCACAGCTGAGGGACAAATTCCCAGCTGACGGGGACCA CAACCTGGATTCCCTGCCCACCCTGGCCATGAGTGCGGGGGCACTGGGAGCTCTACAGCTCCC AGGTGTGCTGACAAGGCTGCGAGCGGACCTACTGTCCTACCTGCGGCACGTGCAGTGGCTGCG CCGGGCAGGTGGCTCTTCCCTGAAGACCCTGGAGCCCGAGCTGGGCACCCTGCAGGCCCGACT GGACCGGCTGCTGCGCCGGCTGCAGCTCCTGATGTCCCGCCTGGCCCTGCCCCAGCCACCCCC GGACCCGCCGGCGCCCCCGCTGGCGCCCCCCTCCTCAGCCTGGGGGGGCATCAGGGCCGCCCA CGCCATCCTGGGGGGGCTGCACCTGACACTTGACTGGGCCGTGAGGGGACTGCTGCTGCTGAA GACTCGGCTGTGA.

Detailed description of the invention:

IL-1 1 is a 19 kDa polypeptide consisting of 178 amino acids, which does not contain potential glycosylation residues, disulphide bonds or other post-translational modifications and has a close similarity to IL-6. It binds to a multimeric receptor complex which contains an IL- 11 specific a-receptor subunit and a promiscuous β subunit (gpl30). IL-1 1 has been demonstrated to improve platelet recovery after chemotherapy-induced thrombocytopenia, induces acute phase proteins, modulates antigen-antibody responses, participates in the regulation of bone cell proliferation and differentiation and could be use as a therapeutic for osteoporosis. Besides from lymphopoietic/hematopoietic and osteotrophic properties, it has functions in many tissues such as brain, gut and testis. IL-1l stimulates the growth of certain lymphocytes and, in the murine model, stimulates an increase in the cortical thickness and strength of long bones.

SD is an acidic protein having low pi of 4.0 present at the N terminus portion of the target protein fused (IL-1 1) having a pi of 11.16. This information can be used for the purification of protein of interest from the tag by using cation exchange chromatography at pH 8.0 wherein the fusion tag, due to its low pi does not bind to the resin whereas protein of interest will bind to the cation exchange resin. In this way, SD can be used as an effective tool to remove the fusion tag after cleavage by simple cation exchange

chromatography. This tag can be fused to any other protein of therapeutic value having a high pi point and similar strategy can be applied for their purification as well for example Interferon Beta pi, 9.69, Nesiritide pi 10.9 etc.

In an embodiment of the invention the protein obtained from the fermentation is further purified by subjecting the fusion protein to hydrophobic interaction chromatography.

Hydrophobic interaction chromatography (HIC) is used as the first step for the purification. There are several resins which can be used for the particular application like butyl sepharose, phenyl sepharose; octyl sepharose etc. The purified fusion protein may further be treated with enterokinase to cleave the fusion protein to obtain the protein of interest. The above digested protein is purified by weak or strong cation exchange chromatography, preferably weak cation exchange chromatography. Several ion exchange resins can be used such as CM sepharose, SP sepharose etc.

In an embodiment of the invention enterokinase from any sources such as bovine enterokinase, human enterokinase, porcine enterokinase or recombinant enterokinase may be used. Recombinant enterokinase may be obtained from bacterial such ***E.coli*** or yeast such as ***Pichia pastoris*** may be used. Enterokinase can then be removed from the IL 11 by the same step of cation exchange chromatography as enterokinase with pi of 5.5 will not bind to cation exchange resin and will come in the flow through along with other protein impurities. On the other hand, target protein binds to the column which can be eluted by giving salt gradient.

In another embodiment, enterokinase used in the process is recovered from the flow-through of cation exchange chromatography by loading it in an affinity chromatography resin (eg. soyabean trypsin inhibitor agarose) Sepharose resin. This way, the IL-11 downstream operations can be made cost effective by recycling enterokinase.

In an another embodiment of the invention the fusion protein may be subjected to ion exchange chromatography followed by HIC followed by enterokinase digestion and further purifying with ion exchange chromatography.

In the purification process carried out by this method IL-1 1 fusion protein is active before and after cleavage with enterokinase and since it is active before cleavage also, this can be exploited to check whether the fusion protein is active or not through out the process.

The advantage of the present invention is that the protein of interest is having a novel fusion tag which imparts solubility to the target protein. Also the protein does not have to go through the complex process of refolding in order to have the biologically active form. In addition to it, the present invention provides a very simple and cost effective process to achieve the purity level of more than 99%.

Definitions

The term fusion tag used herein refers to a fusion tag comprising Serine Aspartate residue sequence, repeated 49 times, having a total of 107 amino acids (having pi of 4.0) for separation of fusion tag from the protein of interest after cleavage using simple and cost effective purification steps. The fusion tag as described provides solubility to the protein and also simplifies the purification of the target protein.

As used herein the term Hydrophobic Interaction Chromatography refers to a separation technique that uses the properties of hydrophobicity to separate proteins from one another. In this type of chromatography, hydrophobic groups such as phenyl, octyl, or butyl, are attached to the stationary column. Proteins that pass through the column that have hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column. In this separation, a buffer with a high ionic strength, usually ammonium sulfate is initially applied to the column. The salt in the buffer reduces the solvation of sample solutes thus as solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium. To elute the proteins, the salt concentration is gradually decreased in order of increasing hydrophobicity. Additionally, elution can also be achieved through the use of mild organic modifiers or detergent.

In an embodiment the hydrophobic interaction chromatography is carried out using a salt concentration of more than 0.5 M.

As used herein the term Enterokinase is a Serine Protease enzyme which converts inactive trypsinogen into active trypsin by cleavage at the C-terminal end of the sequence. It consists of a disulfide-linked 82-140 kDa heavy chain which anchors enterokinase in the intestinal brush border membrane and a 35-62 kDa light chain which is the catalytic subunit. Enterokinase cleaves after lysine at its cleavage site Asp-Asp-Asp-Asp-Lys. It will sometimes cleave at other basic residues, depending on the conformation of the protein substrate. Enterokinase will not cleave at site followed by proline.

As used herein the term Ion Exchange Chromatography relies on charge-charge interactions between the proteins and the charges immobilized on the resin. Ion exchange chromatography can be subdivided into cation exchange chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, in which the binding ions are negative, and the immobilized functional group is positive. Once the solutes are bound, the column is washed to equilibrate it in your starting buffer, which should be of low ionic strength, then the bound molecules are eluted off using a gradient of a second buffer which steadily increases the ionic strength of the eluent solution. Alternatively, the pH of the eluent buffer can be modified as to give the protein or the matrix a charge at which they will not interact and the molecule of interest elutes from the resin.

The invention is further illustrated by the following examples which are provided merely to be exemplary of the invention and do not limit the scope of the invention. Certain modifications and equivalents will be apparent to those skilled in the art and are intended to be included within the scope of the invention.

Example 1: *In vitro* Biological Assay

Cells were cultured in RPMI medium containing 10% fetal calf serum, penicillin (100 units ml^{-1}), streptomycin (1 mg ml^{-1}) and human IL-3 (1 ng ml^{-1}) (Medium A). For cell proliferation assay, cells were pre-incubated in medium A devoid of IL-3 for 3 hours. Cells ($5x10^3$/well/100 µï in Medium A devoid of IL-3) were then incubated with purified IL-1 1 (200 ng ml^{-1}) in 96 well tissue culture plates for 48 hours at 37°C, 5% $C0_2$. MTS (20 µï, Cell Titer 96 Aqueous One Solution, Promega) was added to each well and A_{490}

was monitored after 4 hours of incubation at room temperature. A_{490} of control wells (without IL-11) were subtracted from IL-11 treated wells to get specific absorbance of treated wells.

IL-11 obtained in this manner was seen to be 95 % active when compared with the marketed formulation.

Example 2: Processing the Cell Lysate

Cell lysate of the fusion protein is centrifuged at 15000 rpm for 15 minutes at room temperature. Supernatant is collected and to the supernatant, NaCl is added to the final concentration of 500-2000 mM preferably 1000 mM. When NaCl is completely dissolved, Polyethylene Imine is added with constant stirring from 0.05 % to 5 % concentration, preferably 0.05% to precipitate DNA (both plasmid and host chromosomal DNA) from the cell lysate.

After DNA precipitation, cell lysate is centrifuged at 13 to 15,000 rpm for 15 minutes between 4-25 °C. Supernatant obtained is used as starting material for purification.

Example 3: Purification Using Hydrophobic Interaction Chromatography

The column is pre-equilibrated with a suitable buffer which may include 10-50 mM Tris-Cl pH 8.0, 20-50mM sodium acetate buffer, 50-100 mM sodium phosphate buffer etc. In HIC, protein should bind to the resin at a reasonably high concentration of salt. The salt concentration should be tried depending upon the hydrophobicity of the target protein i.e. 1-2 M NaCl or 0.5-2 M ammonium sulphate can be used. After loading, the column is washed with 5 column volumes equilibration buffer and the bound protein is eluted with 20-50 mM sodium acetate buffer, preferably 50-100 mM sodium phosphate buffer and most preferably with 10-50 mM Tris-Cl, pH 8.0.

Example 4: Enterokinase Cleavage

HIC eluted protein is dialyzed against 10-200 mM, preferably 20 mM Tris pH 8.0 for 16 hours in cold (4-10 °C). To the dialyzed protein, $CaCl_2$ is added to the concentration of 1-10 mM, preferably 2-5mM. Enterokinase is added for the cleavage of fusion protein in the range of 1-5 units of Enterokinase for 400 microgram of the GMSD-IL-11 fusion protein at 10-30 °C, for 16 hours after which, there is almost 90 percent digestion of the fusion

protein. Higher concentrations of EK could also be added for 100% fusion cleavage or by adding the same amount of EK but carrying out the EJK digestion at a higher temperature (RT-40 °C).

Example 5: Purification using Ion Exchange Chromatography

The column is pre-equilibrated with sodium phosphate buffer 20-100mM, pH 7-8, 10-lOOmM Tris, pH 8.0, preferably with 20-50mM Tris, pH 8.0 .After loading, the column is washed with 5 column volumes of equilibration buffer to remove loosely bound impurities. The bound proteins can then be eluted with 20-100mM sodium phosphate buffer , pH 7-8, 20-50 mM Tris pH 7-8.0 containing 100-500mM NaCl.

The CM elution is loaded onto Q sepharose at the conductivity of 20-25 mS/cm , protein of interest comes in the unbound fraction whereas the impurities are bound to the resin and is eluted in 1 M NaCl salt, the unbound protein at this step is >99% pure.

Example 6: Purification using High Performance Liquid Chromatography (HPLC)

Reverse phase HPLC runs were carried out in Shimadzu Model 2010 CHT. The analysis was done on a Vydac C4 4.6*50 50mm, 5μηι column and a 2ml/min flow rate. Gradient chromatography used 0.1% trifluoroacetic acid as buffer A and 0.1% trifluroacetic acid in 90% aqueous acetonitrile as buffer B. A linear gradient of 10 min from 30 to 70% B was used .Samples for analysis were injected. RP-HPLC Profile of in house and Innovator IL-11 was performed. HPLC runs used Shimadzu Model 2010 CHT. The analysis used a Vydac C4 4.6*50 50mm, 5um column and a 2ml/min flow rate. Gradient chromatography used 0.1% trifluoroacetic acid as buffer A and 0.1% trifluroacetic acid in 90% aqueous acetonitrile as buffer B. A linear gradient of 10 min from 30 to 70% B was used. Samples for analysis were injected.

CLAIMS

1. A method for purification of human Interleukin 1l from bacterial cells comprising steps of :

(a) purifying the fusion protein using hydrophobic interaction chromatography,

(b) cleaving the fusion protein with enterokinase, and

(c) purifying IL-1 1 by ion exchange chromatography,

such that the purification steps may be in any order.

2. The method as claimed in claim 1, wherein hydrophobic interaction chromatography is performed using resins selected from butyl sepharose, phenyl sepharose or octyl sepharose.

3. The method as claimed in claim 2, wherein hydrophobic interaction chromatography uses a salt concentration of more than 0.5 M.

4. The method as claimed in claim 1, wherein Ion exchange chromatography is selected from cation exchange and anion exchange in any order.

5. The method as claimed in claim 1, further comprises use one or more fusion tags selected from the group consisting of SD tag, GM tag, T7 tag, GST tag, His tag, Trx Tag or MBP tag.

6. The method as claimed in claim 5, wherein the fusion tag is SD tag comprising nucleotide sequence

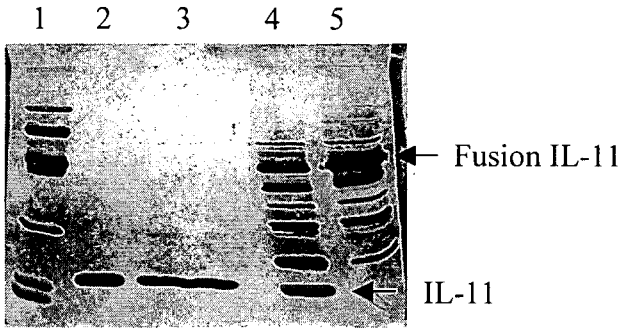
ATGAGCGATTCCGATTCAGACTCGGACTCGGATTCCGATTCCGACAGTGATTCAGATTCTGACT CAGATTCCGATTCTGATTCTGATTCGGATTCCGACTCCGATAGCGACTCAGATAGTGACTCTGA CTCGGACAGCGATTCTGATAGCGACTCTGATTCCGATAGCGATAGCGATTCAGATAGCGATTC TGACTCGGATTCTGATTCCGATTCTGACTCTGACAGCGATTCCGATAGCGACAGCGACTCTGAT AGTGATTCAGACTCTGATTCTGATAGTGATAGCGATTCGGATAGTGGATCCGATGATGATGAT AAA.

7. The method as claimed in claim 5, wherein the fusion tag is SD tag comprising amino acid sequence

MSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSD SDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSGSDDDDK.

8. The method as claimed in claim 4, wherein ion exchange chromatography is cation exchange chromatography.

9. The method as claimed in claim 8, wherein ion exchange chromatography is performed using resins selected from CM sepharose or SP sepharose.



Lane#1: Marker (14 to 97 kDa)
Lane#2: Innovator IL -11
Lane#3: In –house IL-11
Lane#4: EK Digest
Lane#5: Fusion IL-11, undigested

Figure 1. represents SDS-PAGE separation of Interleukin 11 wherein

Lane 1 represents Marker,

Lane 2 represents Innovator IL -11,

Lane 3 represents In –house IL-11,

Lane 4 represents EK Digest and

Lane 5 represents undigested fusion IL 11 protein.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/0O122O

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K1/18 C07K1/20 C07K14/31 C12N15/62
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAVALLI E E R ET AL: "A THIOREDOXIN GENE FUSION EXPRESSION SYSTEM THAT CI RCUMVENTS INCLUSION BODY FORMATION I N THE E. COLI CYTOPLASM" , BIO/TECHNOLOGY, NATURE PUBLISHING CO. NEW YORK, US, vol . 11, no. 2 , 1 February 1993 (1993-02-01) , pages 187-193, XP000195207 , ISSN : 0733-222X, DOI : 10. 1038/NBT0293-187 the whole document	1-9

X	WO 95/16044 A2 (GENETICS INST [US]) 15 June 1995 (1995-06-15) c i ted i n the appl i cati on exampl e 2	1-9

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2011	07/10/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Westphal -Daniel , K

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/0O122O

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 231 217 A2 (GENETICS INST [US] INST GENETICS LLC [US] GENETICS INST LLC [US]) 14 August 2002 (2002-08-14) exampl e 2	1-9
X	EP 1 493 815 Al (INST GENETICS LLC [US]) 5 January 2005 (2005-01-05) exampl e 2	1-9
X	W0 96/38570 Al (GENETICS INST [US]) 5 December 1996 (1996-12-05) exampl e 2	1-9
X, P	W0 2010/125588 Al (LUPIN LTD [IN] ; BANERJEE SAMPALI [IN] ; PADMANABHAN SRI RAM [IN]) 4 November 2010 (2010-11-04) exampl es 1-6; sequence 8	1-9

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2011/0O122O

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9516044 A2	15-06-1995	AU 1399495 A	27-06-1995
EP 1231217 A2	14-08-2002	AT 456661 T	15--02--2010
		AT 237692 T	15--05--2003
		AU 663489 B2	12--10--1995
		CA 2093643 AI	07--08--1992
		DE 69233008 DI	22--05--2003
		DE 69233008 T2	22--01--2004
		EP 1231275 A2	14--08--2002
		EP 0574506 AI	22--12--1993
		JP 2513978 B2	10--07--1996
		JP 5507209 T	21--10--1993
		WO 9213955 AI	20--08--1992
		US 5270181 A	14--12--1993
EP 1493815 AI	05-01-2005	NONE	
W0 9638570 AI	05-12-1996	AT 278785 T	15-·10--2004
		AU 718013 B2	06-·04--2000
		AU 5537896 A	18-·12--1996
		CA 2220447 AI	05-·12--1996
		DE 69633563 DI	11-·11--2004
		DE 69633563 T2	16-·02--2006
		EP 0828842 AI	18-·03--1998
		JP 4011115 B2	21-·11--2007
		JP 11509725 T	31-·08--1999
		US 5760189 A	02-·06--1998
W0 2010125588 AI	04-11-2010	NONE	