

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 June 2008 (05.06.2008)

PCT

(10) International Publication Number
WO 2008/065673 A1

(51) International Patent Classification:

C12N 9/70 (2006.01) **C07K 14/61** (2006.01)
A61K 38/27 (2006.01) **C12N 15/18** (2006.01)
A61K 38/48 (2006.01) **C12N 15/57** (2006.01)
C07K 14/315 (2006.01)

(21) International Application Number:

PCT/IN2007/000012

(22) International Filing Date: 12 January 2007 (12.01.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

2217/CHE/2006 30 November 2006 (30.11.2006) IN

(71) Applicant (for all designated States except US): **BIOCON LIMITED** [IN/IN]; 20th KM, Hosur Road, Electronics City, Bangalore, Karnataka 560 100 (IN).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **SASTRY, Kedar-nath** [IN/IN]; 77a, 2nd Cross, Bannashankri, 3rd Stage, 3rd Phase, 4th Block, Bangalore 560 085, Karnataka (IN). **SRIRAM, Akundi, Venkata** [IN/IN]; 1 A M 3, S F H S Layout, B T M 2nd Stage, Bangalore 560 076, Karnataka (IN). **GOEL, Anuj** [IN/IN]; House No.279, 11th Cross, 29th Main, 1st Phase, JP Nagar, Bangalore 560 078, Karnataka (IN). **KAMATH, Sandeep, Vishwanath** [IN/IN]; 5, Sona Villa, Parsi Colony, Road No.4, Dadar [East], Mumbai 400 014, Maharashtra (IN). **VALERA, Hardik** [IN/IN]; C/o. Mr. R.C. Valera, B/4, "Viram", Gayatri Nagar Society, Near Shrikant Park, Opposite Rameshwar Temple, B/h Hariom Nagar, Vadodara, Gujarat 390 021 (IN). **GARG, Mayank, Kumar** [IN/IN]; S/o. Dr. S.K. Garg, 1093, Moh. Tihai, Mawana, Distt Meerut, Uttar Pradesh 560 401 (IN). **SREENIVAS, Suma** [IN/IN]; #67/1, 7th Cross, Malleswaram, Bangalore, Karnataka

560 003 (IN). **SWETHA, Malur, Dattatreya** [IN/IN]; Kalamandir, 5th Cross, A N Subbarao Road, 4th Main, Hanumanth Nagar, Bangalore, Karnataka 560 019 (IN). **BASU, Amrita** [IN/IN]; # 351, 1 "E" Cross, 3rd Main6th Block, 2nd Phase, Banashankari 3rd Stage, Bangalore, Karnataka 560 085 (IN). **RAGHUNANDAN, Reena, Nichinmetla** [IN/IN]; # 13, Subramanya Swamy Temple Street, Vishweshwara Puram, Bangalore, Karnataka 560 004 (IN).

(74) Agent: **BHOLA, Ravi**; K & S Partners, 4121/b, 6th Cross, 19a Main, Hal II Stage [Extension], Bangalore, Karnataka 560 038 (IN).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report

(54) Title: A METHOD FOR PRODUCING N-TERMINAL METHIOINE FREE POLYPEPTIDES IN MICROBIAL HOST CELLS

(57) Abstract: A method of making N-terminal methionine free polypeptide in a recombinant microbial host cell and pharmaceutical compositions along with one or more pharmaceutical excipients for management of various therapeutic disorders. Specifically, the invention relates to the production of N-terminal methionine free Streptokinase or human growth hormone by co expression with a protease enzyme in *E.coli*.

WO 2008/065673 A1

A METHOD FOR PRODUCING N-TERMINAL METHIOINE FREE POLYPEPTIDES IN MICROBIAL HOST CELLS

Field of the Invention

The present invention provides a method of obtaining a N-terminal methionine free
5 polypeptides in *E.coli* by constructing a plasmid to express a Ubiquitin like protein
SMT3-polypeptide fusion protein in the *E.coli* cytoplasm, which is cleaved by the co-
expressed ULP1 protease. The SMT3-polypeptide fusion protein of the instant
invention is specifically a SMT3-Streptokinase, a SMT3-Streptokinase fusion protein.

Background and prior art of the Invention

10 Streptokinase is a thrombolytic drug approved for use in acute myocardial infarction
and thromboembolism. Streptokinase acts on plasminogen to convert it into plasmin
which in turn degrades fibrin into soluble products. Streptokinase C (SKC) is
synthesized as a precursor protein of 440 amino acids from which a 26 amino acid
signal sequence is cleaved off before secretion from hemolytic Streptococci. Both
15 native (non-recombinant) and recombinant SKC (rSKC) are commercially available.

In two recent studies it was reported that, of the sixteen SKC preparations available in
the market, only three showed potency fulfilling the criteria established by European
Pharmacopoeia (Longstaff C, Thelwell C, and Whitton C., *J. Thromb. Haemost.*, 3:
1092-1093, 2005 and Hermentin P, Cuesta-Linker T, Weisse, Schmidt K-H, Knorst M,
20 Scheid M and Thimme M, *European Heart Journal*, 26:933-940, 2005). Discrepancies
were observed in potency of many marketed rSKC as measured by in vitro assays as
compared to a reference standard. Among the three rSKC samples included in the
study, two showed potency ranging from 20.8% to 37.2% of the label claim, while a
third rSKC preparation from China showed abnormal reaction kinetics and hence
25 potency could not be determined at all. The N-termini of rSKC was found to be
variable. While one rSKC preparation from Cuba showed approximately 50% of
molecules with correct N-termini beginning with isoleucine, while the other 50% still
had intact methionine at N-termini. An rSKC preparation from China had 6 extra amino
acids at the N-terminus, presumably from the signal sequence. The third rSKC from
30 India gave two sequences, both of which had no relationship to SKC N-terminus.
Native SKC has the following amino acid sequence at N-terminus:
IAGPEWLLDRPSVNH.

Currently SKC potency evaluation is done by two in-vitro assays - one in the absence
of fibrin using a chromogenic substrate and another using fibrin as a substrate for clot-

lysis where the activity is declared in international reference units. The value obtained by these two assays is identical when native streptokinase is tested. If the manufacturer of the recombinant SKC choose only one method (either chromogenic or clot-lysis assay) to determine potency, it is possible that the potency values obtained could be either be overestimated or underestimated. This under or over estimation has dramatic consequences during treatment. If the dose administered is lower than recommended, then reperfusion rate could be lower. Where as a high dose of streptokinase administered could cause increase intracranial hemorrhage.

The N-terminus of Streptokinase plays a key role in Plasminogen activation during fibrin clot lysis Mundada LV, Prorok M, DeFord ME, Figuera M, Castellino FJ, Fay WPJ Biol Chem. 2003 Jul 4;278(27):24421-7. We have observed variation in the activity is up to two fold between the two assays for recombinant streptokinases with or without the N-terminal methionine. Thus there is a need to make a good quality recombinant SKC which is methionine free at its N-terminus, herein after called Met⁰ SKC, whose potency as measured by both chromogenic and clot-lysis assay are identical and comparable to properties of native SKC. The method used to manufacture Met⁰ SKC should be also simple, efficient and commercially viable.

When recombinant proteins are expressed in *E.coli*, it is necessary to have ATG codon, encoding Methionine for initiation of protein synthesis. When heterologus proteins are expressed in *E.coli*, the N-terminal methionine may be removed *in vivo*, by methionine amino peptidase encoded by the genome of *E.coli*. In some cases, either due to primary or secondary structure, or inaccessibility of the N-terminus, or simply due to over expression, the N-terminal methionine fails to be removed. One method to solve this problem is to employ a tag at the N-terminus of the recombinant protein with suitable cleavage site to construct a fusion protein. Methods for producing a recombinant protein with an N- or C-terminal tag are well known in the art. For therapeutic applications, the tag is removed from the purified recombinant protein by enzymatic cleavage *in vitro*, to generate the authentic N-terminus bearing protein. Many tags are available to the practitioners of art. An example of such a strategy is adding Glutathione S-transferase (GST) tag ending in C-terminus with amino acids Ile-Glu/Asp-Gly-Arg, which is cleaved specifically by Factor Xa protease, resulting in a recombinant protein with authentic N-terminus. This method is cumbersome as Factor Xa protease has to be produced separately and purified before its use *in vitro* for fusion

protein cleavage. Also, many of the proteases also cleave the fusion protein at various non-canonical sites reducing overall yield of recombinant protein.

The table below summarizes the current state of art, wherein only enterokinase, Factor Xa protease and intein tags allow cleavage in a specific manner, without leaving behind

5 extra amino acids at the N-terminus.

Excision site ↓	Cleavage Enzyme/ Self-Cleavage	Comments
Asp-Asp-Asp-Asp-Lys↓	Enterokinase	*The site will not cleave if followed by a proline residue. Secondary cleavage sites at other basic residues, depending on conformation of protein substrate. Active from pH 4.5 to 9.5 and between 4°C and 45°C.
Ile-Glu/Asp-Gly-Arg↓	Factor Xa protease	Will not cleave if followed by proline and arginine. Secondary cleavage sites following Gly-Arg sequences.
Leu-Val-Pro-Arg↓Gly-Ser	Thrombin	Secondary cleavage sites. Biotinylated form available for removal with immobilized streptavidin.
Glu-Asn-Leu-Tyr-Phe-Gln↓Gly	TEV protease	Seven-residue recognition site, making it a highly site-specific protease. Active over a wide range of temperatures. Protease available as a His-tag fusion, allowing for protease removal after recombinant protein cleavage.
Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro	PreScission™ protease	Genetically engineered form of human rhinovirus 3C protease with a GST fusion, allowing for facile cleavage and purification of GST-tagged proteins along with protease removal after recombinant protein cleavage. Enables low-temperature cleavage of fusion proteins containing the eight-residue recognition sequence.
Specific intein-encoded sequences	Intein 1 & intein 2	Uses self-cleavable affinity tags. Even after cleavage unnatural termini are present on the protein of interest.
Signal sequences	Signal peptidases	Cleavage of leader sequence concomitant with protein export from the cytoplasm.
*LaVallie, E.R., McCoy, J.M., Smith, D.B. & Riggs, P. (1994). Enzymatic and chemical cleavage of fusion proteins. In Current Protocols in Molecular Biology. pp. 16.4.5-16.4.17, John Wiley and Sons, Inc, New York, NY.		

The reason for the ever increasing number of tags made available for various N-terminus cleavages is due to the fact that it is not possible to predict whether a particular tag will provide the desired result. Two common reasons for using a tag are

10 to improve the expression of a recombinant protein or make the expressed recombinant

protein soluble. Often, recombinant heterologous proteins expressed in *E.coli* cytoplasm become insoluble forming the so called "inclusion bodies" and the use of tag may make the protein soluble at least partially. In other cases the tag may make the purification process simpler. For example adding a His tag to the expressed recombinant protein, allows simple and rapid purification using metal affinity chromatography step. One could also combine two tags, for example, His tag (comprising of six or ten Histidine residues) could be fused to another tag (for example, SMT3) which could make the recombinant protein more soluble. The advantage of His tag is easy purification from cytoplasm, using metal affinity chromatography.

A second reason for adding tags to N-terminus is to make the proteins stable and prevent its degradation by proteases in *E.coli* cytoplasm. Small peptides/proteins with low molecular weight (< 10 kD) are often degraded rapidly when expressed by themselves in *E.coli*. For example, small peptides like GLP-1, insulin, etc have been shown to be stable only when they are expressed along with a tag at N-terminus.

Small Ubiquitin related Modifier protein (SUMO), is a protein which is covalently attached post translationally to N-terminus of various proteins. SMT3 from *Saccharomyces cerevisiae* is an example of Small Ubiquitin like protein. Ubiquitin and Ubiquitin like proteins play an important role in various cell functions (nuclear metabolism, cell proliferation, autophagy, etc.) by modulating protein structure and function. ULP1 protease releases SMT3 tag from proteins to which it is attached. Interestingly prokaryote like *E.coli* has neither SMT3 nor ULP1 protease gene in its genome. Hence use of SMT3 tag in prokaryotes is much more convenient. There are also no proteases described in *E.coli* which could cleave SMT3. Also, ULP1 protease, due to its high specificity can not cleave any *E.coli* protein and is thus not toxic to *E.coli*. Hence use of SMT3 tag in *E.coli* is much easier as compared to eukaryote like yeasts, in which there is a protease which cleaves SMT3 tag in the middle, after which due to alteration in three dimensional structure of the SMT3 protein; the C-terminal half SMT3 attached to a protein of interest fails to be cleaved by ULP1 protease, unless one adds back N-terminal half SUMO to the cleavage reaction.

Ubiquitin-like-specific protease 1 (ULP1) is deubiquitinating enzyme which cleaves the C-termini of SMT3 and deconjugate SMT3 from the side chains of lysines (Li, S.J. and Hochstrasser, M. (1999) *Nature* 398, 246–251). Three-dimensional structure of a complex between SMT3 and ULP1 show that certain surface features of SMT3 are

recognized by ULP1 that make the interaction specific (Mosesso, E. and Lima, C.D. (2000) *Mol. Cell.* **5**, 865–876). The catalytic domain (amino acids 403-621) of ULP1 is sufficient for deconjugation of SMT3 from fusion proteins and has been shown to be functional when expressed in *E.coli* (Mosesso, E. and Lima, C.D. (2000) *Mol. Cell.* **5**, 865–876).

Objects of the present invention:

The principal object of the present invention is to produce N-terminal methionine free polypeptides in microbial host cells.

10 Another object of the present invention is to produce N-terminal methionine free polypeptides in bacterial and fungal host cells.

Yet another object of the present invention is to produce N-terminal methionine free streptokinase in *E.coli* host cells.

Still another object of the present invention is to develop a method for the making of
15 N-terminal methionine free polypeptides in microbial host cells.

Still another object of the present invention is to develop a method for the making of N-terminal methionine free streptokinase in *E.coli* host cells.

Still another object of the present invention is to arrive at pure form of recombinant N-terminal methionine free streptokinase (Met⁰ SKC).

20 Still another object of the present invention is to prepare a pharmaceutical composition comprising streptokinase (Met⁰ SKC) along with one or more pharmaceutically acceptable excipients.

Still another object of the present invention is to prepare a pharmaceutical composition comprising Met⁰ hGH along with one or more pharmaceutically acceptable excipients.

25

Statement of Invention:

A method of making N-terminal methionine free polypeptide in a recombinant microbial host cell comprising: (i) preparing an expression construct containing a DNA encoding a polypeptide of interest; (ii) preparing an expression construct containing a
30 DNA encoding a protease enzyme (iii) cloning both the expression constructs of (i) and (ii) into the host cell to obtain transformant; (iv) fermenting the transformant and inducing co-expression; and (v) obtaining the N-terminal methionine free polypeptide; a method of making N-terminal methionine free streptokinase in *E.coli* host

comprising: (i) preparing an expression construct containing a DNA encoding a SUMO-streptokinase fusion protein; (ii) preparing an expression construct containing a DNA encoding a Ulp1 protease; (iii) cloning both the expression construct of (i) and (ii) into an *E.coli* host; (iv) fermentation of the transformants and inducing co-expression; and (v) obtaining the N-terminal methionine free streptokinase by lysing the cells and purifying the protein; a substantially pure recombinant N-terminal methionine free streptokinase (Met⁰ SKC); a pharmaceutical composition comprising streptokinase (Met⁰ SKC) which is substantially free of Met-SKC along with one or more pharmaceutically acceptable excipients; and a pharmaceutical composition comprising Met⁰ hGH which is substantially free of N-terminal methionine-hGH along with one or more pharmaceutically acceptable excipients.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1. Map of the plasmid with SKC coding sequence driven by a T7 promoter sequence

Fig.2. SDS-PAGE analysis of total cell lysate from induced and uninduced cultures of *E.coli* BL21 (DE3) transformed with pET SKC plasmid.

Fig.3. Map of the plasmid with mutated version of *E.coli* MAP coding sequence driven by native promoter.

Fig.4. SDS-PAGE analysis of total cell lysate from induced and uninduced cultures of *E.coli* BL21 (DE3) transformed with pET ULP1 plasmid.

Fig.5. Schematic diagram showing method of construction of plasmid for co-expression of *Saccharomyces cerevisiae* ULP1 in *E.coli*

Fig.6. Schematic representation of SMT3 and SKC gene fusion

Fig.7 is a map of the plasmid with SMT3-SKC fusion coding sequence driven by a T7 promoter sequence

Fig.8. SDS-PAGE analysis of total cell lysates from induced and uninduced cultures of *E.coli* BL21 (DE3) co-transformed with pET SKC and pTrcULP1/pACYC plasmids.

Fig:9 Illustration explaining the soluble chromogen assay and clot lysis assay.

Description of Certain Preferred Embodiments of the Invention

The present invention is in relation to a method of making N-terminal methionine free polypeptide in a recombinant microbial host cell comprising:

- (i) preparing an expression construct containing a DNA encoding a polypeptide of interest;
- (ii) preparing an expression construct containing a DNA encoding a protease enzyme
- 5 (iii) cloning both the expression constructs of (i) and (ii) into the host cell to obtain transformant;
- (iv) fermentatng the transformant and inducing co-expression; and
- (v) obtaining the N-terminal methionine free polypeptide.

Another embodiment of the present invention wherein the microbial host cell is
10 selected from a group comprising bacteria and fungi.

Yet another embodiment of the present invention wherein N-terminal methionine free polypeptide is btained either by secretion or by lysing the cells and purifying the protein.

Still another embodiment of the present invention wherein the polypeptide of interest is
15 a hormone, an enzyme or a therapeutic protein.

Still another embodiment of the present invention wherein the hormone is a growth hormone, luteinising hormone, or a natriuretic peptide

Still another embodiment of the present invention wherein the therapeutic enzyme is streptokinase

20 Still another embodiment of the present invention wherein the growth hormone is hGH
The present invention is in relation to a method of making N-terminal methionine free streptokinase in *E.coli* host comprising:

- (i) preparing an expression construct containing a DNA encoding a SUMO-streptokinase fusion protein;
- 25 (ii) preparing an expression construct containing a DNA encoding a Ulp1 protease;
- (iii) cloning both the expression construct of (i) and (ii) into an *E.coli* host;
- (iv) fermentation of the transformants and inducing co-expression; and
- (v) obtaining the N-terminal methionine free streptokinase by lysing the cells and purifying the protein.

30 Another embodiment of the present invention wherein the *E.coli* host is a BL21 or BL26 strain

Yet another embodiment of the present invention wherein the expression construct is a pET vector containing a DNA encoding SMT3-Streptokinase fusion protein.

Still yet another embodiment of the present information, wherein the expression construct^v is a pACYC184 vector containing a DNA encoding Ulp1 protease operationally linked to a promoter.

Still yet another embodiment of the present information wherein the DNA encoding
5 Ulp1 protease operationally linked to a promoter is integrated into *E.coli* genome.

Still yet another embodiment of the present information wherein N-terminal methionine free streptokinase is Met⁰ SKC of Seq. ID no. 17, with potency identical to native SKC. The present invention is in relation to a substantially pure recombinant N-terminal methionine free streptokinase (Met⁰ SKC)

10 The present invention is in relation to a pharmaceutical composition comprising streptokinase (Met⁰ SKC) which is substantially free of Met-SKC along with one or more pharmaceutically acceptable excipient

The present invention is in relation to a pharmaceutical composition comprising Met⁰ hGH which is substantially free of N-terminal methionine-hGH along with one or more
15 pharmaceutically acceptable excipients.

Another embodiment of the present invention, wherein the pharmaceutical excipients are selected from a group comprising granulating agents, binding agents, lubricating agents, disintegrating agents, sweetening agents, coloring agents, flavoring agents, coating agents, plasticizers, preservatives, suspending agents, emulsifying agents and
20 spheronization agents.

Recombinant SKC when expressed in *E.coli* without a tag at the N-terminus results in a mixture of molecules with and without Methionine. The physicochemical properties of the rSKC molecules with and without Methionine are very similar and are hence difficult to separate by simple techniques. In the current invention, we have genetically
25 engineered a SMT3 tag at the N-terminus of SKC to generate fusion protein, which is cleaved by the co-expressed ULP1 protease resulting in Met⁰ SKC with correct N-terminus, matching that of native streptokinase. A vector has been developed in which PCR amplified SMT3 coding sequence has been fused in frame with PCR amplified coding sequence of mature SKC (Met⁰ SKC) and cloned into a vector in which the
30 fusion protein sequence is operationally linked to phage T7 promoter. This recombinant vector is cloned to express the recombinant SMT3-Met⁰ SKC fusion protein in a genetically engineered *E.coli* host like BL21 (DE3) or BL26 (DE3) strains, upon induction with isopropyl-beta-D-thiogalactopyranoside (IPTG). The recombinant

SMT3-SKC fusion protein is expressed into the *E.coli* cytoplasm and is cleaved by the by co-expressed ULP1 *in vivo*. The ULP1 protease very specifically recognizes the three dimensional features of SMT3 protein, in addition to Gly-Gly sequence, in the C-terminus and cleaves the SMT3 to release Met⁰ SKC. This simplifies the method of cleavage as opposed to *in vitro* cleavage which requires a second fermentation to produce ULP1 protease. The ULP1 protease is expressed in the same cell by a compatible, low copy number recombinant plasmid vector into which PCR amplified coding sequence of ULP1 protease is cloned operationally linked to *tac* promoter which is also inducible by IPTG.

10 The instant invention allows a simple method of producing Met⁰ SKC in large quantities using recombinant techniques for use in pharmaceutical compositions. The alternate methods described in literature involve over expression of *E.coli* gene coding for methionine amino peptidase (MAP). MAP over expression does not always help in removal of methionine from the N-terminus for various reasons like the nature of penultimate amino acid, secondary structure of the protein near the N-terminus, etc. It has been previously reported that if the amino acid next to initiator methionine is Ala, Gly, Pro and Ser, it is easily cleaved by MAP. If the penultimate amino acid is Ile, Val, Cys or Thr, then there is variability in cleavage and removal of methionine. Finally, if the penultimate amino acid is Lys, Arg, Leu, Asp, Asn, Phe, Met, Trp, Tyr, Glu, Gln or His, the initiator methionine is not removed. (Ben-Bassat A, Bioprocess Technology 12:147-159, 1991). In the case of SKC, penultimate amino acid is Isoleucine and hence we also found variability in removal of methionine by MAP

25 A recent report (Liao YD, Jeng JC, Wang CF, Wang SC, Chang ST., Protein Science. 13:1802-1810, 2004, US Patent 7109015) suggested that site-directed mutagenesis of three residues in *E.coli* methionine amino peptidase substrate binding pocket (Y168G, M206T, Q233G) allowed the removal methionine from proteins even if the penultimate residues were Met, His, Asp, Asn, Glu, Gln, Leu, Ile, Tyr, and Trp. However, in the case of SKC, neither over expression of the native MAP nor over expression of the mutated *E.coli* MAP (with Y168G, M206T & Q233G amino acid substitutions) failed to remove methionine from N-terminus of Met SKC completely, even though penultimate residue is Ile. SKC there fore appears to be unique protein, removal of methionine from which appears to be a challenge. Because of these constraints, we

30

sought an alternate method in which close to 100% of the recombinant SKC molecules produced would be methionine free.

SMT3 coding sequence was PCR amplified from genomic DNA of *Saccharomyces cerevisiae*. SKC mature form coding nucleic acid fragment was amplified from the genomic DNA of *Streptococcus equisimilis*. By employing overlapping primers, the C-terminal end of SMT3 (ending in amino acids Gly-Gly) was fused in frame to N-terminal of Met⁰ SKC (beginning with amino acids Ile-Ala). This fused PCR product was cloned into pET vector using which recombinant proteins could be made by induction with IPTG after transformation into appropriate commercially available genetically engineered *E.coli* host like BL21 (DE3), into whose genome lacUV5 promoter driven T7 phage polymerase coding sequence has been integrated (Novagen, Madison, USA). PCR technique was used to amplify ULP1 protease gene from *Saccharomyces cerevisiae* genome and cloned into a low copy number vector (pACYC) vector. The expression of ULP1 was driven by *tac* promoter, which is also induced by IPTG. An alternative method of co-expression of ULP1 protease by integration of the ULP1 gene into *E.coli* genome is also possible. Preferably the ULP1 gene could be targeted to *msbB* gene coding sequence, the disruption of which could cause a reduction in the levels of endotoxin from the *E.coli* host, helping in improving the quality of therapeutic protein by reduction of endotoxin impurity.

The technology of the instant Application is further elaborated with the help of following examples. However, the examples should not be construed to limit the scope of the invention.

Example 1

Cloning and Expression of rSKC in E.coli.

Preparation of replicative plasmid for intracellular production of Streptokinase in *E.coli*

Amplification of SKC gene:

The source of DNA for mature Streptokinase encoding gene was the genomic DNA isolated from *Streptococcus* sp. strain BICC #7869 (MTCC 389). Genomic DNA was isolated using a commercially available (Qiagen) kit following manufacturer's instructions. The following primers were used for the amplification of the SKC gene.

SKC FP1: 5' CAT ATG ATT GCT GGA CCT GAG TGG CTG CTA 3' (Seq. ID#1)

SKC RP1: 5' CGG GAT CCT TAT TTG TCG TTA GGG TTT ATC AGG 3' (Seq. ID#2).

Proof reading *Pwo* polymerase (Roche) was used for PCR and the SKC gene was amplified. The amplified product was electrophoresed on 1% agarose gel and was purified using the gel extraction kit (Qiagen). The gel purified product was modified to have dATP overhangs and this product was ligated to pTZ57R/T vector (MBI Fermentas). The ligation reaction was carried out at 16°C overnight. The ligation mix was transformed to chemically competent *E.coli* DH5 α cells and plated on LB Ampicillin 100 μ g/ml plates. The transformants were screened by isolating plasmid from 1 ml of culture by mini lysis method and was digested with restriction enzymes BamHI and XbaI (NEB) to release the insert. The digestion was carried out at 37°C for 2 hours. One positive clone was selected and inoculated to LB medium containing Ampicillin 100 μ g/ml and plasmid was isolated by Qiagen mini prep kit and the plasmid was sequenced to confirm the presence of the gene and any possible mutations. The sequence confirmed plasmid was then used for further sub-cloning.

Sub cloning SKC coding DNA fragment to pET vector: The plasmid which has the SKC gene in pTZ57R/T vector was digested with the restriction enzymes NdeI and BamHI and electrophoresed on 1% agarose gel. The insert released was excised and gel eluted and ligated to pET vector which was also digested with the same enzymes. The ligation was done using the enzyme T4 DNA ligase (NEB) and ligation was carried out at 16 °C overnight. The ligation mix is used to transform competent *E.coli* DH5 alpha cells and the transformants were selected on LB plates with 100 μ g/ml Ampicillin. The transformants are screened for the presence of the SKC gene. The correct clone (Fig. 1) was mapped and confirmed and this plasmid is used for transforming competent *E.coli* BL26 (DE3) host.

Expression in *E.coli* BL26 (DE3) host: The verified plasmid clone was transformed to *E.coli* BL26 (DE3) host and the transformants were selected on Ampicillin (100 μ g/ml) plates. A few transformants were grown in LB Ampicillin (100 μ g/ml) liquid media and when the OD at 600nm is ~0.6 to 0.8 the cells are induced with IPTG (1 mM). The induction is carried out overnight at 30 °C and the cells were pelleted and resuspended in buffer and sonicated to lyse the cells. The samples were loaded on SDS-PAGE. The results are shown in Fig. 2. A good level of induction was observed. Recombinant SKC

was purified and sequencing of the product showed that only 40% of the rSKC molecules were Met⁰ SKC.

Example 2.

5 *Over expression of E.coli MAP in E.coli expressing SKC*

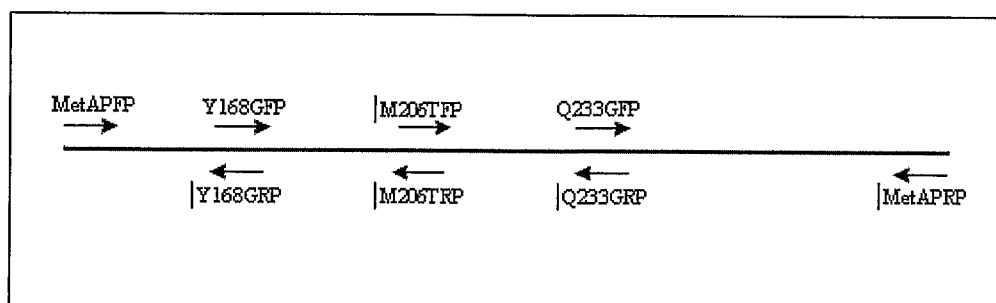
The main goal of this experiment was to determine if over expression of MAP in the same cell as that expressing rSKC would help in efficient removal of N-terminal methionine.

E.coli MAP gene was amplified from BL21 (DE3) host genomic DNA using the
10 following primers (Seq. ID# 3 to Seq. ID# 10) to generate a mutated version which has been reported to remove N-terminal methionine more efficiently from proteins with Ile as the penultimate amino acid (Liao YD, Jeng JC, Wang CF, Wang SC, Chang ST., Protein Science. 13:1802-1810, 2004, US Patent 7109015). The mutations introduced were 168Y, 206M, 233Q.

Y168GFP	5'TTCGTGAAGGCTGCGGACACGGTATTGGTCGCGG3'	Seq. ID# 3
Y168GRP	5'GTCCGCAGCCTTCACGAACGACGGAGAAGCCTTC3'	Seq. ID# 4
M206TFP	5'CGAGCCAACCGTCAACGCGGGTAAAAAAGAGATC3'	Seq. ID# 5
M206TRP	5'CGTTGACGGTTGGCTCGATGGTGAACGTCATCCC3'	Seq. ID# 6
Q233GFP	5'GTCTGCAGGCTATGAGCATACTATTGTGGTGACTG3'	Seq. ID# 7
Q233GRP	5'GCTCATAGCCTGCAGACAAGCTGCGATCTTTGG3'	Seq. ID# 8
MetAPFP	5'GGATCCGACGTCGAATTTTCTATTA3'	Seq. ID# 9
MetAPRP	5'TCGGGTATAGCTATGAAAGCAGCTG3'	Seq. ID# 10

15

The primer positions have been represented in the following figure.



The source of DNA for Methionine amino peptidase (MAP) was *E.coli* genomic DNA
20 from BL21 (DE3) strain which was isolated by routine protocols (Molecular Cloning: A Laboratory Manual By Joseph Sambrook, David W Russell, 2001, CSHL Press).

Four separate PCR reactions were performed using *E.coli* genomic DNA as template and the proof-reading polymerase, *Pwo* polymerase with various primer pairs as outlined in the following table:

Primer pairs	Size of amplified product
MetAPFP/ Y168GRP	510bp
Y168GFP/M206TRP	132bp
M206TFP/Q233GRP	96bp
Q233GFP/MetAPRP	310bp

- 5 The four PCR reactions were run on 1.2% agarose gel for 1 hr at 100 mV. Individually the amplified fragments were excised from the gel and purified using Qiagen gel extraction kit. To get full length MAP gene (1048kb), all the four fragments were used as template using MetAPFP & MetAPRP primers. Proof reading *Pwo* polymerase (Roche) was used for PCR and the full length MAP gene was amplified.
- 10 The amplified product was electrophoresed on 1% agarose gel and was purified using the gel extraction kit (Qiagen). The gel purified product was modified to have dATP overhangs and this product was ligated to pTZ57R/T vector (MBI Fermentas). The ligation reaction was carried out at 16°C overnight. The ligation mix was transformed to chemically competent *E.coli* DH5 α cells, plated on LB Ampicillin (100 μ g/ml) plates
- 15 incubated at 37 °C, overnight. The transformants were screened by isolating plasmid from 1 ml of culture by mini lysis method and was digested with restriction enzymes BamHI and XbaI (NEB) to release the insert. The digestion was carried out at 37°C for 2 hours. One positive clone was selected and inoculated to LB broth containing Ampicillin (100 μ g/ml), incubated at 37 °C shaker incubator, overnight. Plasmid was
- 20 isolated by Qiagen mini prep kit and the plasmid was sequenced to confirm the presence of the gene and mutations made at three positions.. The sequence confirmed plasmid was then used for further sub-cloning.

- Sub-cloning MAP (Seq. ID #18) gene to pACYC vector:** The plasmid with the MAP gene in pTZ57R/T vector was digested with the restriction enzymes SalI followed by
- 25 BamHI and was electrophoresed on 1% agarose gel. The insert released was excised from the gel, eluted and ligated to pACYC vector which was also digested with the same enzymes sequentially. The ligation was done using the enzyme T4 DNA ligase (NEB) and ligation was carried out at 16 °C overnight. The ligation mix was used to

transform competent *E.coli* DH5 α cells and the transformants were selected on LB plates with Chloramphenicol (15 μ g/ml). The transformants were screened for the presence of the MAP gene. The correct clone was mapped and confirmed (Fig. 3).

- 5 **Expression in *E.coli* BL26 (DE3) host:** The verified plasmid clone was co-transformed along with pET SKC vector to *E.coli* BL26 (DE3) host and the transformants were selected on Ampicillin (100 μ g/ml) and Chloramphenicol (15 μ g/ml) plates. A few transformants were grown in LB broth with Ampicillin (100 μ g/ml) and Chloramphenicol (15 μ g/ml). When the OD at 600 nm was ~0.6 to 0.8
- 10 the cells were induced with IPTG (1 mM). The induction was carried out overnight at 30 °C and the cells were pelleted and resuspended in buffer and sonicated to lyse the cells. The samples were loaded on SDS-PAGE.

After studying the expression of rSKC (Seq. ID # 17) and purification, the results indicated that there was no improvement in efficiency of removal of methionine from

15 N-terminus (data not shown). Approximately 40%-60% of the rSKC molecules still had methionine at their N-terminus.

Example 3:

Cloning and Expression of SMT3-SKC fusion in *E.coli*

The genomic DNA of *Saccharomyces cerevisiae* strain (BICC# 7806) was isolated

20 using standard techniques. This was used as the template and a PCR reaction was carried out using the primers SMT FP1 (5' CATATGGGTCATCACCATCATCATCACGGGTCGG ACTCAGAAGTC 3', Seq. ID# 11) and SMT RP1 (5'CTCAGGTCCAGCAATACCTC CAATCTGTTCGCGGTG 3', Seq. ID# 12), the SMT-3 gene was amplified. The reaction mix was electrophoresed

25 on 1.2% agarose gel. The amplified product was checked for the size and was gel eluted and was used as the template for overlapping PCR. In a similar way using the genomic DNA of the strain BICC 7869 and the primers SMT-SKC FP1 (5' GAACAGATTGAAGGTATTGCTGGACCTGAGTGGCTG 3', Seq. ID# 13) and SMT-SKC RP1 (5'CGG GAT CCT TAT TTG TCG TTA GGG TTT ATC AGG 3',

30 Seq. ID# 14) the SKC gene was amplified by PCR using proof reading polymerase. This fragment was also gel eluted and used as template for overlapping PCR. By mixing both the purified PCR products and using them as the template, PCR was performed to get the full length overlapped product. This is electrophoresed on 1%

agarose gel and the product is gel eluted. This is then ligated to pTZ57R/T vector by creating dATP overhangs and the ligation was carried out at 16°C overnight. The ligation mix is then transformed to competent *E.coli* DH5α cells and the mix was plated on LB containing Ampicillin (100µg/ml) plates and the plates were incubated at 37°C incubator overnight. The transformants which appear were screened for the presence of the gene and this plasmid was purified by using Qiagen mini prep kit. This plasmid was sequenced to confirm the overlap and for mutations. The confirmed plasmid was used for further experiments.

This plasmid was digested with the restriction enzymes NdeI and BamHI (NEB) and the reaction mix was incubated at 37°C for 2 hours. The reaction was terminated by heat inactivation of the enzymes and it was electrophoresed on 1% agarose gel. The insert released was gel eluted and was used for ligation with the vector. The vector is prepared by digesting pET vector with the enzymes NdeI and BamHI and processed in a similar way as done to the insert. Then a ligation reaction was set using T4 DNA ligase (NEB) and the reaction was carried out at 16°C overnight. The ligation mix was then transformed to competent *E.coli* DH5α cells and then selected on LB Kanamycin (25µg/ml) plates. The transformants were screened for the presence of the gene and the plasmid was isolated from the correct clone using Qiagen Mini prep kit. The plasmid was confirmed by restriction analysis and was then used for expression studies.

Schematic representation of SMT3 AND SKC gene fusion is illustrated in Fig: 6. The plasmid containing SMT3-SKC fused gene (Fig: 7) product in pET vector was used to transform competent *E.coli* BL21 (DE3) cells. The transformants were selected on LB containing Kanamycin (25µg/ml) plates. A few colonies were inoculated to 100 ml LB containing Kanamycin (25µg/ml) and were grown to OD 600 of ~0.7-0.8. A small sample was removed as control and the rest of the culture was induced with IPTG to a final concentration of 1 mM. The induction was performed overnight at 30°C with shaking at 120 rpm. The cells were then pelleted and resuspended in buffer and sonicated to lyse the cells and the samples were analyzed on 10% SDS-PAGE.

Example 4:

30 Cloning and expression of Ulp1 protease in *E.coli*

Using the primers Ulp1 FP (NdeI) 5' CAT ATG CTT GTT CCT GAA TTA AAT GAA AAA GAC G 3' (Seq. ID# 15) and Ulp1 RP (XhoI) 5' CTC GAG TTT TAA AGC GTC GGT TAA AAT C 3' (Seq. ID# 16), the ULP1 (Seq. Id # 19) gene was

- amplified by PCR using *Saccharomyces cerevisiae* genomic DNA as the template (BICC# 7806) and proof reading Pwo polymerase (Roche) enzyme. The PCR product was gel purified and cloned to pTZ57R/T vector (MBI Fermentas) after creating dATP overhangs. The ligation was carried over night at 16 °C. The ligation mix was
- 5 transformed to competent *E.coli* DH5 α cells and selected on LB Ampicillin (100 μ g/ml) plates. The colonies were screened by PCR using M13 forward and M13 reverse primers and gene specific forward and reverse primers. One correct clone was selected and plasmid from this clone was isolated by using Qiagen mini prep method. This clone was sequenced to confirm the presence of the gene and any possible mutations
- 10 Results of sequencing of the PCR product showed three mutations. All three mutations were silent mutations (Asp⁴¹³ GAC to GAT, Ser⁴⁷³ TCA to TCG, Glu⁵⁵⁴ GAG to GAA). The correct confirmed clone was digested with restriction enzymes NdeI and XhoI (NEB) and the insert released was gel purified and ligated to the pET vector which was also digested with NdeI and XhoI restriction enzymes. The ligation was done using the
- 15 enzyme T4 DNA ligase (NEB) and the reaction was carried out at 16°C overnight. The ligation mix was then transformed to competent *E.coli* DH5 α cells by heat shock method. The transformation mix was then plated on LB plates carrying Kanamycin (25 μ g/ml) and the plates were incubated at 37°C overnight. The Kanamycin resistant transformants were screened for the presence of the gene and the correct clone was
- 20 inoculated to LB medium with Kanamycin (25 μ g/ml) and plasmid was isolated by using Qiagen mini prep kit.
- After confirming the authenticity of the plasmid by restriction digestions, it was transformed to chemically competent *E.coli* BL21 (DE3) competent cells and was plated on LB Kanamycin (25 μ g/ml) plates. The plates were incubated at 37 °C
- 25 overnight. The Kanamycin resistant transformants were checked for protein production. A few Kanamycin resistant transformants were inoculated to 100 ml of liquid LB broth containing Kanamycin (25 μ g/ml) and this was grown at 37°C till the OD at 600 nm reached 0.8. At this stage the culture was induced and a small amount of sample was removed as control. Induction was done with IPTG (1 mM) and the culture was further
- 30 grown at 30 °C overnight. After overnight IPTG induction, the OD at 600 nm was checked and the cells were pelleted by centrifugation at 6000 rpm for 6 minutes. The cells were then resuspended in a buffer (100 mM Tris buffer, pH 8.0 with 0.1M NaCl) and sonicated using Branson sonifier till the OD dropped to 1/5 -1/8 of the initial OD.

The soluble fraction was loaded on to Ni-NTA agarose matrix.(Qiagen) which was equilibrated with the same buffer. The matrix was washed with the same buffer and the protein was eluted from the matrix using the same buffer supplemented with 250 mM Imidazole. The results are shown in Fig. 5. A good level of induction (and Ulp1 production) was observed and the His-tagged Ulp1 was purified on Ni-NTA column chromatography.

Example 5:

Co-expression of SMT3-SKC fusion protein and ULP1 protease in *E.coli* (Seq Id # 20)

10 Sub cloning of Ulp1 to pTrc99A

The Ulp1 protease encoding DNA fragment which was cloned to pET vector was digested with the restriction enzymes NdeI and BsrBI (NEB) and the digestion is carried out by incubating the reaction mix at 37 °C. The reaction mix was then heated to high temperature to inactivate the enzymes and electrophoresed in 1% agarose gel. The fragment released is then gel eluted and is ligated to pTrc99A vector which was also digested with the enzymes NdeI and SmaI and was treated in similar manner. The ligation reaction was carried out using T4 DNA ligase (NEB) and the reaction was carried out at 16 °C overnight. The ligated sample was then used to transform chemically competent *E.coli* DH5α cells and plated on LB Ampicillin (100g/ml) plates. The transformants which were resistant to Ampicillin (100g/ml) were screened for the presence of the insert by PCR using the gene specific primers. One correct clone was identified and used for further experiments.

Sub cloning Ulp1 coding sequence to pACYC184 vector:

The Ulp1 gene which was cloned to pTrc99A vector was digested with enzymes PvuII and BamHI (NEB) and the digestion was carried out at 37 °C for 2 hours. The reaction was stopped by heat inactivating the enzymes and the mix was electrophoresed on 1% agarose gel. The insert released was gel purified and used for ligation with the vector. The pACYC184 vector was prepared by digesting with enzymes EcoRV and BamHI. The restriction enzyme digested pACYC184 vector was purified in a similar manner (Fig: 8). The insert and the vector were ligated using T4 DNA ligase. The ligated sample was transformed to competent DH5α cells and plated on LB agar plates containing Chloramphenicol (10μg/ml). The plates were incubated at 37 °C overnight.

The Chloramphenicol resistant transformants were screened and the correct clone was confirmed by restriction analysis. This plasmid was used for further experiments.

This pACYC184 vector with insert of Ulp1 gene in was co-transformed along with the plasmid harboring SMT3-SKC gene to chemically competent *E.coli* BL21 (DE3) host and plated on LB plates with Chloramphenicol (10 g/ml) & Kanamycin (25 g/ml). The plates were incubated at 37°C overnight. The transformants which appeared were resistant to both the antibiotics indicating the presence of both the plasmids. A few transformants were inoculated to 100 ml of LB medium containing both the antibiotics and the flask was incubated on a shaker at 37 °C. When the OD at 600 nm reached ~0.7-0.8, a small sample was removed as control and the rest of the culture was induced with IPTG to a final concentration of 1 mM. The culture was next incubated at 30 °C with shaking at 120 rpm overnight. The OD at 600 nm of the culture at the end of induction was checked and the cells were pelleted by centrifugation. The cell pellet was resuspended in a buffer containing 20 mM Tris, pH 8.0 and 100 mM NaCl. After resuspension the OD at 600 nm was checked and the volume was adjusted to ~20-25 OD and the cells were sonicated using a cell disruptor, keeping on ice so that the temperature does not increase. The samples were loaded on 10% SDS-PAGE gel (Fig. 4). The cleavage was very efficient when both the protease and SMT3-SKC (Seq Id #21) were co expressed in the same host. The SKC was purified from lysed *E.coli* cells by standard purification methods. N-terminus of all the purified rSKC molecules was confirmed to be free of methionine.

Met SKC and Met free SKC were purified and analyzed for biological activity in solution chromogenic assay and Fibrin clear clot assay (Illustrated in Fig:9). The third international reference standard for Streptokinase (NIBSC Batch # 00/464 derived from Native Streptokinase) was used to calculate the relative potency and activity. The ratio of the activity for the two streptokinase preparations by these two methods was calculated and is presented in the table below

Batch number	Met SKC	Met free SKC
Batch #1	2.0	1.06
Batch #2	2.5	1.01
Batch #3	1.7	0.91

Seq. ID#1

CAT ATG ATT GCT GGA CCT GAG TGG CTG CTA

Seq. ID#2

CGG GAT CCT TAT TTG TCG TTA GGG TTT ATC AGG

Seq. ID#3

TTC GTG AAG GCT GCG GAC ACG GTA TTG GTC GCG G

5

Seq. ID#4

GTCCGCAGCCTTCACGAACGACGGAGAAGCCTTC

Seq. ID#5

CGAGCCAACCGTCAACGCGGGTAAAAAAGAGATC

10 Seq. ID#6

CGTTGACGGTTGGCTCGATGGTGAACGTCATCCC

Seq. ID#7

GTCTGCAGGCTATGAGCATACTATTGTGGTGACTG

Seq. ID#8

15 GCTCATAGCCTGCAGACAAGCTGCGATCTTTGG

Seq. ID#9

GGATCCGACGTCGAATTTTCTATTA

Seq. ID#10

TCGGGTATAGCTATGAAAGCAGCTG

20 Seq. ID#11

CATATGGGTCATCACCATCATCATCACGGGTCGGACTCAGAAGTC

Seq. ID#12

CTCAGGTCCAGCAATACCTCCAATCTGTTCGCGGTG

Seq. ID#13

25 GAACAGATTGAAGGTATTGCTGGACCTGAGTGGCTG

Seq. ID#14

CGG GAT CCT TAT TTG TCG TTA GGG TTT ATC AGG

Seq. ID#15

CAT ATG CTT GTT CCT GAA TTA AAT GAA AAA GAC G

30 Seq. ID# 16

CTC GAG TTT TAA AGC GTC GGT TAA AAT C

Seq. ID# 17

Amino acid sequence of mature rSKC

1 IAGPEWLLDR PSVNNSLVVS VAGTVEGTNQ DISLKFFEID LTSRPAHGGK
 51 TEQGLSPKSK PFATDSGAMS HKLEKADLLK AIQEQLIANV HSNDDYFEVI
 101 DFASDATITD RNGKVYFADK DGSVTLPTQP VQEFLLSGHV RVRPYKEKPI
 151 QNQAQSV DVE YTVQFTPLNP DDDFRPGLKD TKLLKTLAIG DTITSQELLA
 5 201 QAQSILNKNH PGYTIYERDS SIVTHDNDIF RTILPMDQEF TYRVKNREQA
 251 YRINKKSGLN EEINNTDLIS EKYYVLKKGE KPYDPFDRSH LKLFTIKYVD
 301 VDTNELLKSE QLLTASERNL DFRDLYDPRD KAKLLYNNLD AFGIMDYTLT
 351 GKVEDNHDDT NRIITVYMGK RPEGENASYH LAYDKDRYTE EEREVYSYLR
 401 YTGTPIPDNP NDK

10

Seq. ID# 18

AMINO ACID SEQUENCE OF *E.coli* Methionine aminopeptidase (MAP) WITH
 THREE MUTATIONS (Bold and underlined).

15 1 MAISIKTPED IEKMRVAGRL AAEVLEMIEP YVKPGVSTGE LDRICNDYIV
 51 NEQHAVSACL GYHGYPKSVC ISINEVVCHG IPDDAKLLKD GDIVNIDVTV
 101 IKDGFHGDTS KMFIVGKPTI MGERLCRITQ ESLYLALRMV KPGINLREIG
 151 AAIQKFVEAE GFSVVREGCG HGIGRGFHEE PQVLHYDSRE TNVVLKPGMT
 201 FTIEPTVNAG KKEIRTMKDG WTVKTKDRSL SAGYEHTIVV TDNGCEILTL
 20 251 RKDDTIPAI SHDE

Seq. ID# 19

Nucleotide sequence of the ULP1 PCR product amplified using *Saccharomyces cerevisiae* genome as template. The PCR product amplified nucleotides encoding
 25 amino acids 403-621 of ULP1 protein. The silent nucleotide substitutions are shown in bold and underlined.

1 CATATGCTTG TTCCTGAATT AAATGAAAAA GACGATGATC AAGTACAAAA
 51 AGCTTTGGCA TCTAGAGAAA AACTCAGTT AATGAATAGA GATAATATAG
 101 AGATAACAGT ACGTGATTTT AAGACCTTGG CACCACGAAG ATGGCTAAAT
 30 151 GACACTATCA TTGAGTTTTT TATGAAATAC ATTGAAAAAT CTACCCCTAA
 201 TACAGTGGCG TTAAATTCGT TTTTCTATAC CAATTTATCA GAAAGGGGTT
 251 ATCAAGGCGT CCGGAGGTGG ATGAAGAGAA AGAAGACACA AATTGATAAA
 301 CTTGATAAAA TCTTTACACC AATAAATTG AACCAATCCC ACTGGGCGTT
 351 GGGCATAATT GATTAAAAA AGAAACTAT AGGTTACGTA GATTCATTAT
 35 401 CGAATGGTCC AAATGCTATG AGTTTCGCTA TACTGACTGA CTTGCAAAAA
 451 TATGTTATGG AAGAAAGTAA GCATACAATA GGAGAAGACT TTGATTTGAT
 501 TCATTTAGAT TGTCCGCAGC AACCAAATGG CTACGACTGT GGAATATATG
 551 TTTGTATGAA TACTCTCTAT GGAAGTGCAG ATGCGCCATT GGATTTTGAT

601 TATAAAGATG CGATTAGGAT GAGAAGATTT ATTGCCCAT T GATTTTAAC
651 CGACGCTTTA AAAC TCGAG

Seq. ID# 20

- 5 Amino acid sequence of the ULP1 gene fragment expressed in *E.coli*:

1 MLVPELNEKD DDQVQKALAS RENTQLMNRD NIEITVRDFK TLAPRRWLND
51 TIEFFMKYI EKSTPNTVAF NSFFYTNLSE RGYQGVRRWM KRKKTQIDKL
101 DKIFTPINLN QSHWALGIID LKKKTIGYVD SLSNGPNAMS FAILTDLQKY
151 VMEESKHTIG ĖDFDLIHLDC PQQPNGYDCG IYVCMNTLYG SADAPLDFDY
10 201 KDAIRMRRFI AHLILTDALK LEHHHHHHHHH

Seq. ID# 21

Amino acid sequence of SMT3-SKC fusion protein. The sequence of SMT3 is shown in bold letters.

15 1 MGHHHHHHGS DSEVNQEAKP EVKPEVKPET HINLKVSDGS SEIFFKIKKT
51 TPLRRLMEAF AKRQ GKEMDS LRFLYDGIRI QADQAPEDLD MEDNDIIEAH
101 **REQIGGI**AGP EWLLDRPSVN NSQLVSVAG TVEGTNQDIS LKFFEIDLTS
151 RPAHGGKTEQ GLSPKSKPFA TDSGAMSHKL EKADLLKAIQ EQLIANVHSN
201 DDYFEVIDFA SDATITDRNG KVFYADKDGS VTLPTQPVQE FLLSGHVRVR
20 251 PYKEKPIQNQ AKSVDVEYTV QFTPLNPDDD FRPGLKDTKL LKTLAIGDTI
301 TSQELLAQAQ SILNKNHPGY TIYERDSSIV THDNDIFRTI LPMDQEFTYR
351 VKNREQAYRI NKKSGLNEEI NNTDLISEKY YVLKKGEKPY DPFDRSHLKL
401 FTIKYVDVDT NELLKSEQLL TASERNLDFR DLYDPRDKAK LLYNNLDAFG
451 IMDYTLTGKV EDNHDDTNRI ITVYMGKRPE GENASYHLAY DKDRYTEEER
25 501 EVYSYLRYTG TPIPDNPNDK

We Claim:

1. A method of making N-terminal methionine free polypeptide in a recombinant microbial host cell comprising:
 - (i) preparing an expression construct containing a DNA encoding a polypeptide of interest;
 - (ii) preparing an expression construct containing a DNA encoding a protease enzyme
 - (iii) cloning both the expression constructs of (i) and (ii) into the host cell to obtain transformant;
 - (iv) fermentatng the transformant and inducing co-expression; and
 - (v) obtaining the N-terminal methionine free polypeptide.
2. The method as claimed in claim 1, wherein the microbial host cell is selected from a group comprising bacteria and fungi.
3. The method as claimed in claim 1, wherein N-terminal methionine free polypeptide is obtained either by secretion or by lysing the cells and purifying the protein.
4. The method in claim (i) of 1, wherein the polypeptide of interest is a hormone, an enzyme or a therapeutic protein.
5. The method in claim 2, wherein the hormone is a growth hormone, luteinising hormone, or a natriuretic peptide
6. The method in claim 4, wherein the therapeutic enzyme is streptokinase
7. The method in claim 4, wherein the growth hormone is hGH
8. A method of making N-terminal methionine free streptokinase in *E.coli* host comprising:
 - (i) preparing an expression construct containing a DNA encoding a SUMO-streptokinase fusion protein;
 - (ii) preparing an expression construct containing a DNA encoding a Ulp1 protease;
 - (iii) cloning both the expression construct of (i) and (ii) into an *E.coli* host;
 - (iv) fermentation of the transformants and inducing co-expression; and

- (v) obtaining the N-terminal methionine free streptokinase by lysing the cells and purifying the protein.
9. The method according to claim 8, wherein the *E.coli* host is a BL21 or BL26 strain.
- 5 10. The method according to claim (i) of 8, wherein the expression construct is a pET vector containing a DNA encoding SMT3-Streptokinase fusion protein.
11. The method according to claim (ii) of 8, wherein the expression construct is a pACYC184 vector containing a DNA encoding Ulp1 protease operationally linked to a promoter.
- 10 12. The method according to claim (ii) of 8, wherein the DNA encoding Ulp1 protease operationally linked to a promoter is integrated into *E.coli* genome.
13. The method according to claim (v) of 8, wherein N-terminal methionine free streptokinase is Met⁰ SKC of Seq. ID no. 17, with potency identical to native SKC.
- 15 14. A substantially pure recombinant N-terminal methionine free streptokinase (Met⁰ SKC).
15. A pharmaceutical composition comprising streptokinase (Met⁰ SKC) which is substantially free of Met-SKC along with one or more pharmaceutically acceptable excipients.
- 20 16. A pharmaceutical composition comprising Met⁰ hGH which is substantially free of N-terminal methionine-hGH along with one or more pharmaceutically acceptable excipients.
17. The pharmaceutical compositions as claimed in claim 15 and 16, wherein the pharmaceutical excipients are selected from a group comprising granulating agents, binding agents, lubricating agents, disintegrating agents, sweetening agents, coloring agents, flavoring agents, coating agents, plasticizers, preservatives, suspending agents, emulsifying agents and spheronization agents.
- 25

Figure: 1

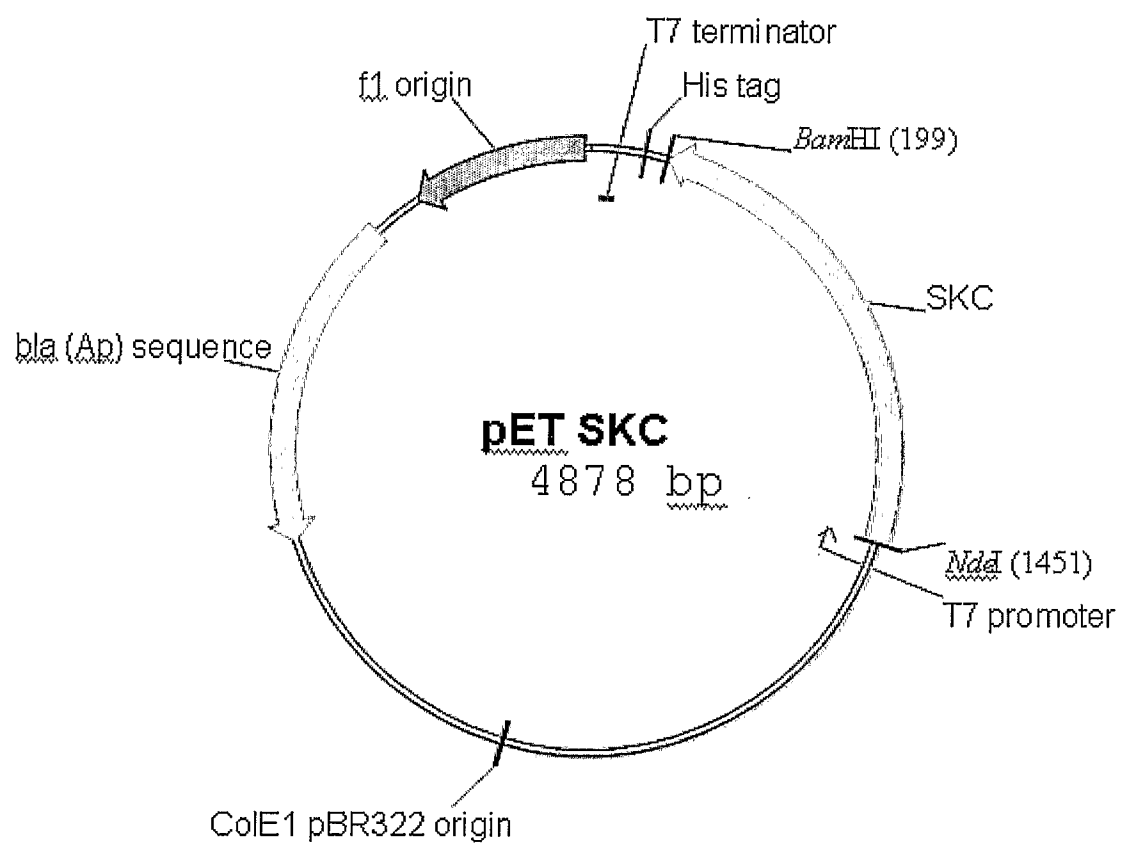


Figure: 2

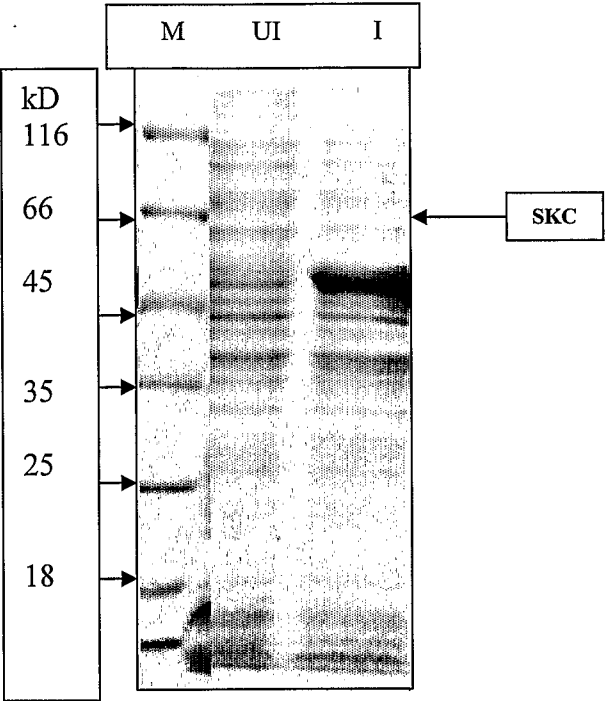


Figure: 3

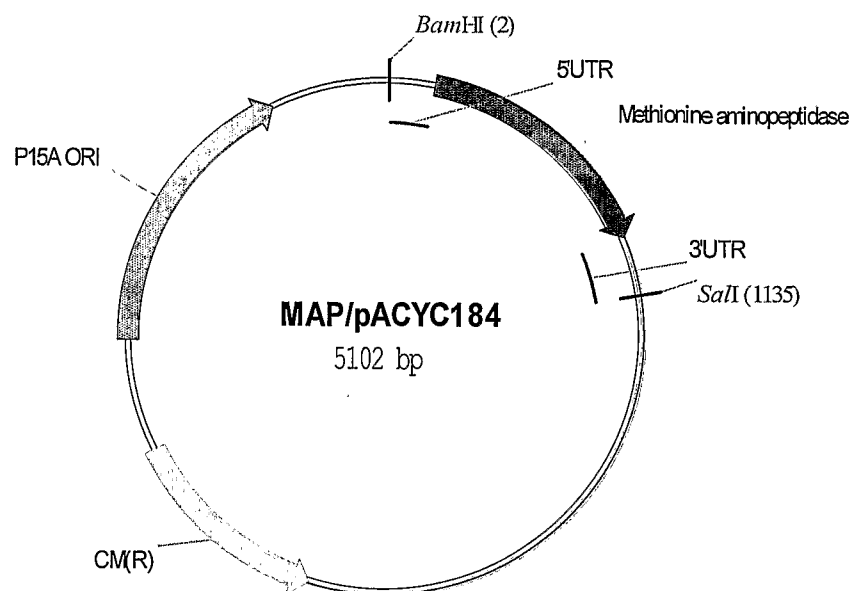


Figure: 4

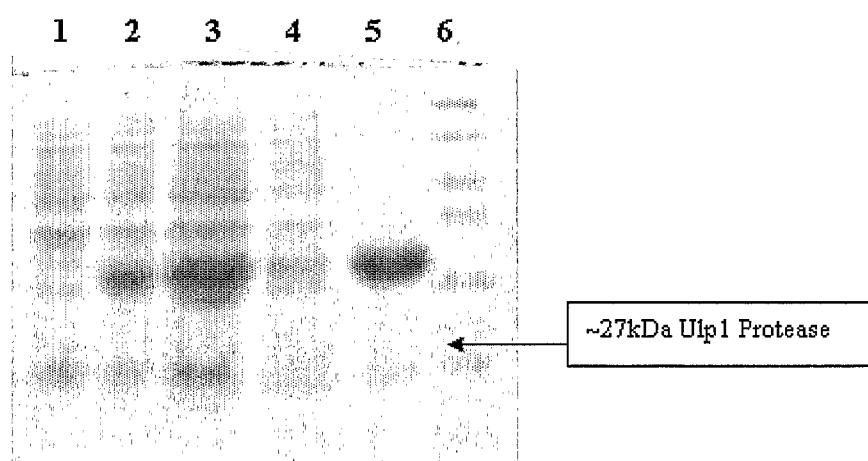


Figure: 5

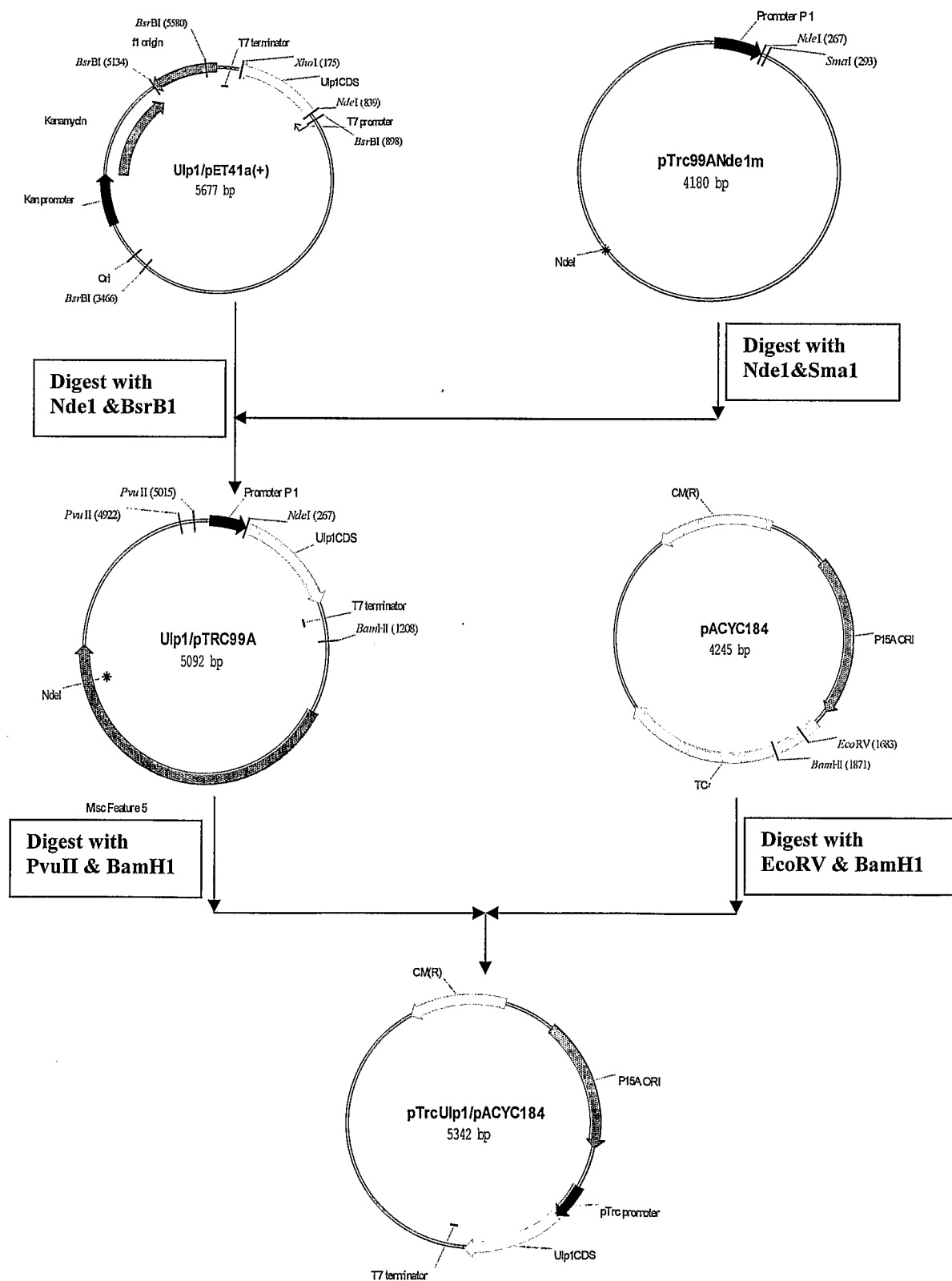


Figure: 6

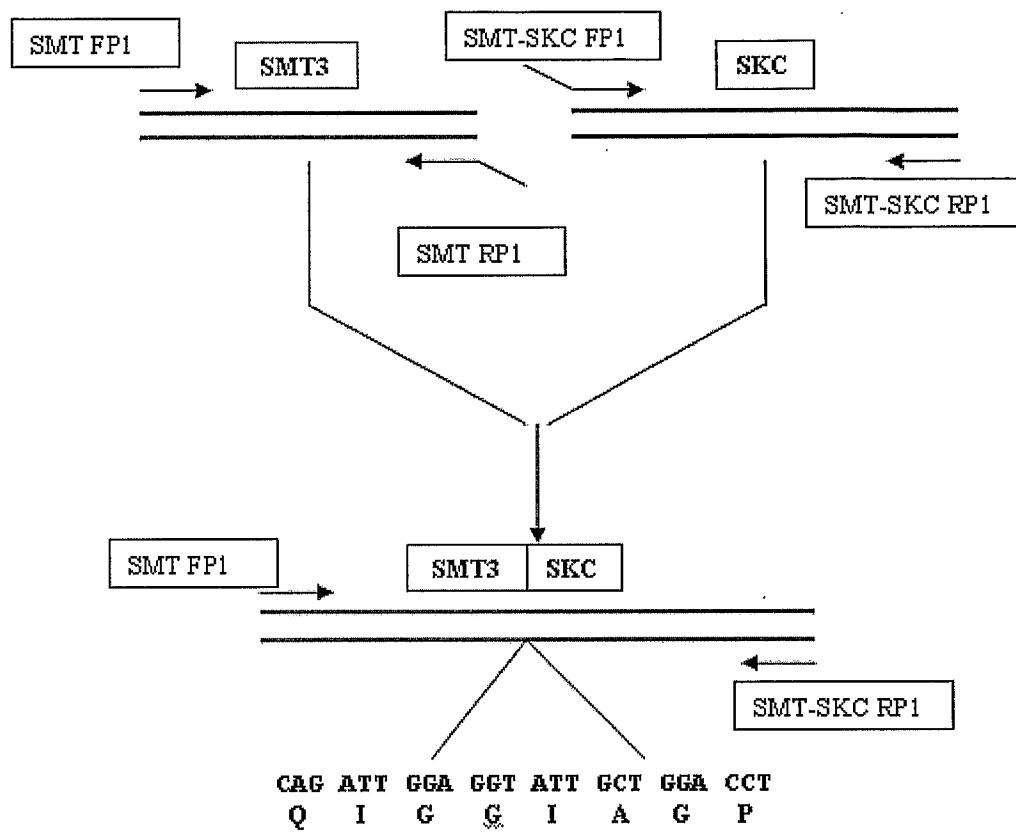


Figure: 7

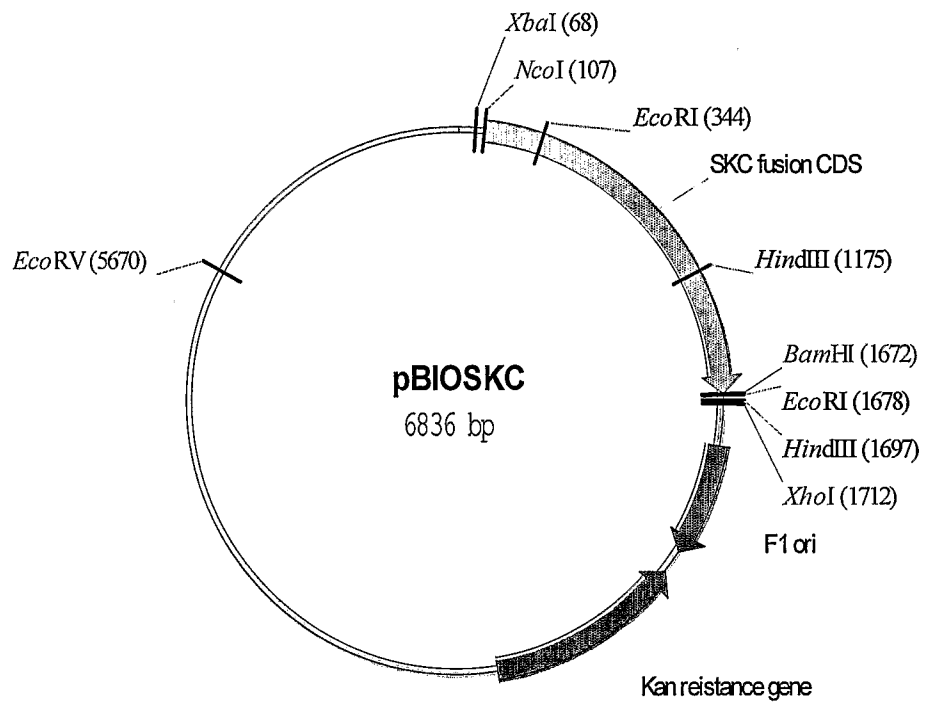


Figure: 8

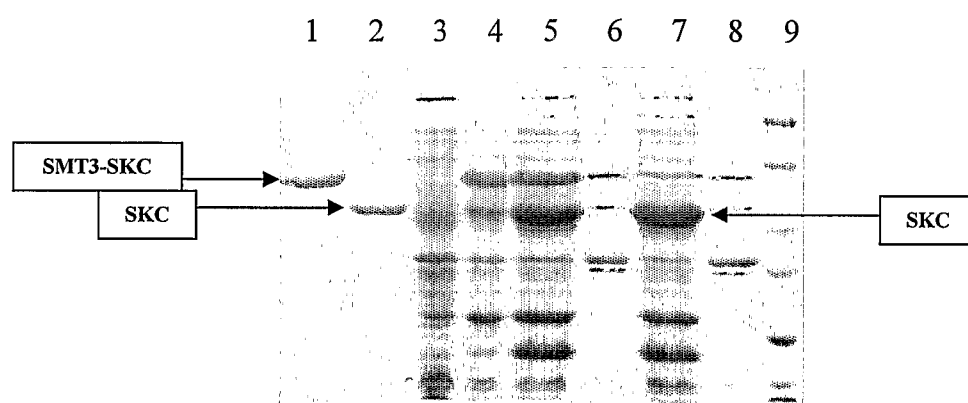
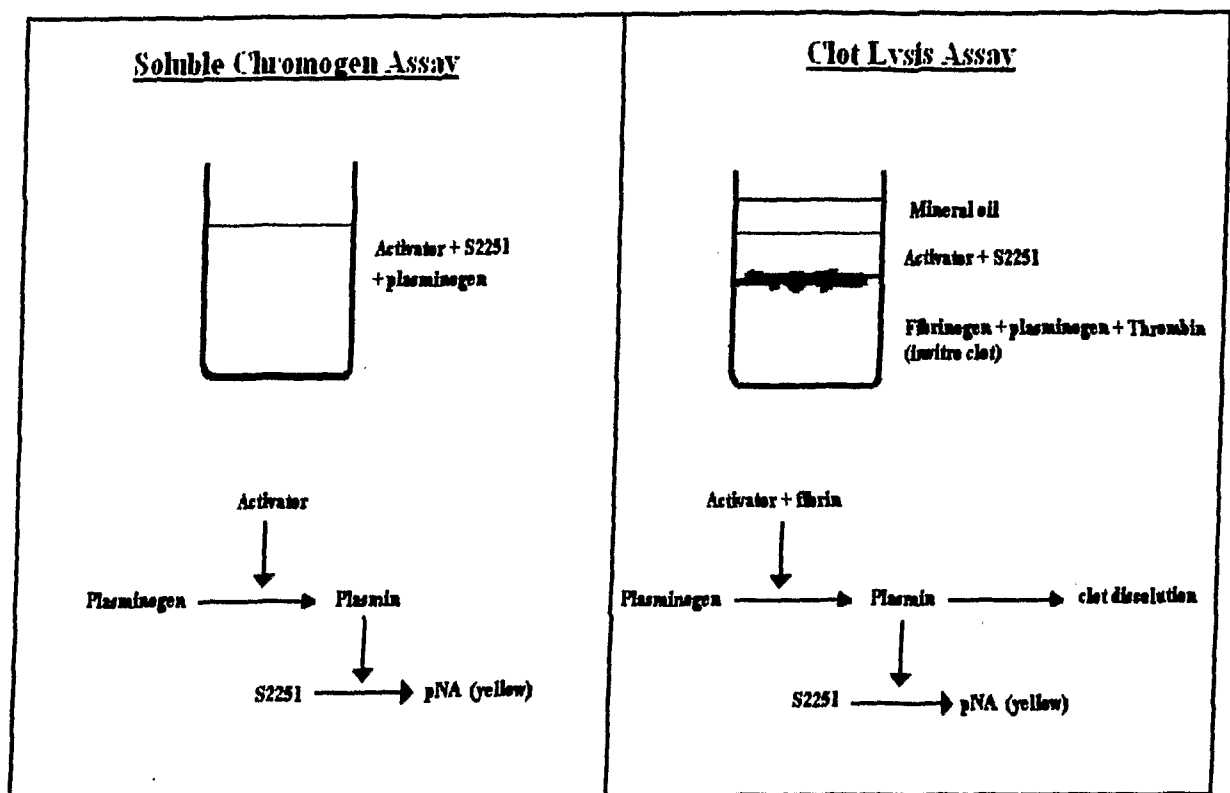


Figure 9



INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2007/000012

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 9/70 (2006.01) C07K 14/315 (2006.01) C12N 15/57 (2006.01)

A61K 38/27 (2006.01) C07K 14/61 (2006.01)

A61K 38/48 (2006.01) C12N 15/18 (2006.01)

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)

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 practicable, search terms used)

STN (BIOSIS, WPIDS, MEDLINE, CA); KEYWORDS: streptokinase, growth hormone, methionine, protease, enzyme

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Liao Y.D. et al., "Removal of N-terminal methionine from recombinant proteins by engineered E.coli methionine aminopeptidase," Protein Science, 2004, 13: 1802-1810. See whole document, in particular abstract, column 1 - page 1803, <i>Applications in other recombinant proteins</i> - page 1805.	1-7
X	US 7,109,015 B2 (YOU-DI LIAO) 29 September 2005 See whole document, in particular example 6, claim 23.	1-7

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
23 April 2007Date of mailing of the international search report
7 MAY 2007Name and mailing address of the ISA/AU
AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustalia.gov.au
Facsimile No. (02) 6285 3929Authorized officer
MELANIE METZ
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No : (02) 6283 2551

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2007/000012

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	US 5,763,215 A (SHMARYAHU BLUMBERG) 9 June 1998	
X	See whole document, in particular examples I, V, XIII.	16, 17
	Franchi, E., et al., "A new human growth hormone production process using a recombinant Bacillus subtilis strain," Journal of Biotechnology, 1991, 18:41-54.	
X	See whole document.	16, 17
	EP 0 629 695 B1 (LUCKY LTD) 21 December 1994	
X	See whole document.	16, 17
	WO 1998/038290 A1 (LG CHEMICAL LTD) 3 September 1998	
X	See whole document, in particular example 12 and claims.	16, 17
	Hermentin, P., et al., "Comparative analysis of the activity and content of different streptokinase preparations," European Heart Journal, 2005, 26:933-940	
X	See whole document, in particular abstract, table 1.	14, 15 and 17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IN2007/000012

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
US	7109015	US	2005214899		
US	5763215	AU	47739/85	EP	0191827
		HK	143096	IL	76107
		WO	1986/001229	LU	91064
EP	0629695	ZA	8506194		
		BR	9402461	CN	1098742
		JP	7147979	EG	21684
US	5569598				
WO	1998/038290	AU	61224/98	BR	9807616
		EP	0975742	PL	335459
				CA	2282734
				US	6428997
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					