(12) STANDARD PATENT (11) Application No. **AU 2008275828 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Method for production of recombinant human thrombin `644

(51) International Patent Classification(s)
C12N 9/64 (2006.01) ***C12P 21/02*** (2006.01)
C12N 9/74 (2006.01)

(21) Application No: **2008275828** (22) Date of Filing: **2008.07.04**

(87) WIPO No: **WO09/008821**

(30) Priority Data

(31) Number	(32) Date	(33) Country
60/948,207	**2007.07.06**	**US**

(43) Publication Date: **2009.01.15**
(44) Accepted Journal Date: **2012.09.27**

(71) Applicant(s)
MedImmune Limited

(72) Inventor(s)
Lovgren, Ann;Harrysson, Anna

(74) Agent / Attorney
Davies Collison Cave, 1 Nicholson Street, Melbourne, VIC, 3000

(56) Related Art
EP 1405910 A1 & EMBL Accession No. BD189686
US 6413737 B1
NISHIDA, S., et al., Biochemistry, 1995, vol. 34, pages 1771-1778 & EBI Accession No. Q90945
US 2004/0197858 A1
YONEMURA, H., et al., Journal of Biochemistry, 2004, vol. 135, pages 577-582

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 January 2009 (15.01.2009)

PCT

(10) International Publication Number
WO 2009/008821 A1

(51) International Patent Classification:
C12N 9/64 (2006.01) *C12P 21/02* (2006.01)
C12N 9/74 (2006.01)

(21) International Application Number: PCT/SE2008/050836

(22) International Filing Date: 4 July 2008 (04.07.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/948,207 6 July 2007 (06.07.2007) US

(71) Applicant (*for all designated States except US*): ASTRAZENECA AB [SE/SE]; S-151 85 Södertälje (SE).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): HARRYSSON, Anna [SE/SE]; AstraZeneca R & D Mölndal, S-43183 Mölndal (SE). LÖVGREN, Ann [SE/SE]; AstraZeneca R & D Mölndal, S-43183 Mölndal (SE).

(74) Agent: ASTRAZENECA AB; AstraZeneca Intellectual Property, S-151 85 Södertälje (SE).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, 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, LY, MA, MD, ME, 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, ST, 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, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2009/008821 A1

(54) **Title:** METHOD FOR PRODUCTION OF RECOMBINANT HUMAN THROMBIN ‘644

(57) **Abstract:** The present invention relates to a method for producing recombinant human thrombin from recombinant prothrombin using recombinant ecarin having an amino acid sequence that is encoded by the nucleotide sequence SEQ ID NO 2.

METHOD FOR PRODUCTION OF RECOMBINANT HUMAN THROMBIN '644

TECHNICAL FIELD

The present application relates to a method for producing recombinant human thrombin from recombinant prothrombin using recombinant ecarin.

BACKGROUND OF THE INVENTION

Thrombin is a key enzyme in the coagulation cascade. By thrombin mediated proteolytic digestion of fibrinogen into fibrin monomer, a cascade reaction leading to clot formation is started. Clot formation is the first step in wound healing. In addition thrombin is a chemo attractant to cells involved in wound healing, and, the fibrin network formed act as a scaffold for collagen-producing fibroblasts, increases phagocytosis, promotes angiogenesis and binds growth factors thus further supporting the healing process. The rate of clot formation is dependent on the concentration of thrombin and fibrinogen. Because of the important function in clot formation thrombin has been utilised in a number of products intended for haemostasis and/or as tissue sealants or "glues", both as stand-alone products (i.e. Thrombin-JMI) or in combination with fibrin or other compounds (i.e. Tisseel, Hemaseel, Crosseal). The potential fields of use are numerous; skin grafting, neuro surgery, cardiac surgery, toracic surgery, vascular surgery, oncologic surgery, plastic surgery, ophthalmologic surgery, orthopedic surgery, trauma surgery, head and neck surgery, gynecologic and urologic surgery, gastrointestinal surgery, dental surgery, drug delivery, tissue engineering and dental cavity haemostasis.

So far the thrombin in approved thrombin-containing products on the market is derived either from human or bovine plasma. Using plasma derived protein confers several disadvantages as limited availability and safety concerns such as risk for transmission of viruses and prions and the risk of triggering autoantibody formation (bovine products). Cases where antibody formation due to bovine thrombin exposure has lead to significant bleeding disorders are known.

In vivo thrombin is obtained from activation of prothrombin through the coagulation cascade. Activation through the coagulation cascade is dependent on the presence of a functional GLA-domain containing 8-10 glutamic residues converted to gamma-carboxyglutamate. *In vitro,* also incomplete gamma-carboxylated prothrombin can be converted to thrombin by the use of prothrombin activators such as ecarin. Ecarin, a snake venom derived from the Kenyan viper *Echis carinatus* is a procoagulant, a protease which cleaves human prothrombin between residues Arg_{320}-Ile_{321} to generate meizothrombin. Further autocatalytic processing results in the formation of meizothrombin desF1 and then alpha-thrombin, which is the mature active form of thrombin.

An ideal commercial thrombin manufacturing process would use a recombinant thrombin precursor and a recombinant protease produced at high productivity without addition of animal-derived components. Further requirements would be robust performance, convenience and low cost.

A big obstacle for efficient recombinant human thrombin (rh-thrombin) has been to obtain high yields of prothrombin. Although extensive efforts have been spent, obtaining high yields of prothrombin under conditions suitable for production of biologicals has long remained a challenge. Yonemura et al. (J Biochem 135:577-582, 2004) have used recombinant GLA-domain-less prethrombin digested with recombinant ecarin to generate recombinant human thrombin. The productivity of prethrombin at process scale was 150-200 mg/L, which is a modest productivity for commercial scale production. Recombinant production of ecarin has also been described in WO 01/04146. In this publication generation of rh-thrombin is exemplified by conversion of recombinant prothrombin produced in COS cells by a recombinant ecarin produced from CHO cells. However, the exemplified methods are not suitable for large-scale production and animal-derived components are used.

Recombinant ecarin is produced as a prepro-protein that needs to be activated. Problems to efficiently activate the r-ecarin are described in both publications and the suggested activation procedures are far from optimal.

2008275828 24 Aug 2012

Thus there is a need for improved methods to obtain recombinant human thrombin. During our efforts to obtain improved productivity of gamma-carboxylated human prothrombin we made the surprising discovery that co-expression with gamma-glutamyl carboxylase (GGCX) vastly improved also the productivity of incompletely carboxylated prothrombin (see WO2005038019).

The present invention describes a process to efficiently produce human thrombin from recombinant prothrombin obtained by the expression method as described in WO2005038019. Recombinant carboxylated or incompletely carboxylated prothrombin combined with recombinant ecarin has not previously been used for manufacturing of recombinant thrombin. Further, the procedure for activating recombinant ecarin is new. The methods described would be suitable for large scale rh-thrombin manufacturing without the addition of animal-derived components.

SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a method comprising:

(a) providing mammalian cells comprising DNA encoding prepro-ecarin comprising SEQ ID NO: 5;

(b) expressing the prepro-ecarin in the cells by incubating the cells under conditions permitting the cells to express the prepro-ecarin from the DNA;

(c) continuing to incubate the cells until substantially all of the cells have ceased to be viable, thereby producing a medium comprising dead cells; and

(d) continuing to incubate the medium for an activation period sufficient to produce active ecarin from the prepro-ecarin in the medium.

According to another aspect of the invention, a method is provided for producing recombinant human thrombin from recombinant prothrombin using recombinant ecarin having the sequence SEQ ID NO 2 or a homologue thereof.

2008275828 25 May 2012

According to another aspect, a pharmaceutical composition is provided comprising a recombinant thrombin according to said method, in combination with pharmaceutically acceptable carriers, vehicles and/or adjuvants.

According to a further aspect, an isolated DNA sequence is provided coding for recombinant ecarin according to SEQ ID NO 2 or a homologue thereof, having at least 80% identity to SEQ ID NO 2.

According to another aspect, a vector is provided comprising an isolated DNA sequence coding for recombinant ecarin according to SEQ ID NO 2 or a homologue thereof, having at least 80% identity to SEQ ID NO 2.

According to yet another aspect, a cell line is provided comprising a vector comprising an isolated DNA sequence coding for recombinant ecarin according to SEQ ID NO 2 or a homologue thereof, having at least 80 % identity to SEQ ID NO 2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. FII+GGCX construct

Figure 2. Ecarin construct

Figure 3. Example of a process outline for thrombin manufacturing

Figure 4. Nucleotide sequence alignment of recombinant ecarin used in the present invention and wild type ecarin

Figure 5. Amino acid alignment of recombinant ecarin used in the present invention and wild type ecarin

Figure 6. Graph showing the activation of recombinant ecarin during cell death over time

Figure 7. Activation of recombinant ecarin in cell cultures over time, assayed by SDS-PAGE

Figure 8. Chromatogram from CIEX purification of rh-thrombin.

Figure 9. Non-reduced SDS-PAGE analyses of fractions obtained by CIEX purification

Detailed description of the invention

The invention consists in one part of a cell line derived by stable transfection with a vector (Figure 1) encoding human prothrombin (FII) associated by suitable control sequences and human gamma-glutamyl carboxylase (GGCX) associated by suitable control sequences. Control sequences should be chosen so that prothrombin expression is in excess of the GGCX expression by at least a factor of 10. The host cell is preferably a eukaryotic cell. Typical host cells include, but are not limited to insect cells, yeast cells, and mammalian cells. Mammalian cells are particularly preferred. Suitable mammalian cells lines include, but are not limited to, CHO, HEK, NS0, 293, Per C.6, BHK and COS cells, and derivatives thereof. In one embodiment the host cell is the mammalian cell line CHO-S. The obtained prothrombin producing cell line is grown under culture conditions optimised for high yield of prothrombin disregarding gamma-carboxylation. Vitamin K may or may not be added to the growth medium.

It will be appreciated that the invention is not restricted to a particular prothrombin or gamma-glutamyl caboxylase or protein encoding sequence of one of these proteins to be co-expressed. Moreover, and in particular with respect to blood coagulation factors, numerous mutant forms of the proteins have been disclosed in the art. The present invention is equally applicable to prothrombin and gamma-glutamyl caboxylase mutant forms, including naturally occurring allelic variants, of the proteins as it is to wild-type sequence. In one embodiment the invention can be undertaking with any wild-type protein or one with at least 90%, preferably at least 95% sequence identity thereto. In another embodiment, sequences listed in Table 1 can be used.

Table 1

PROTEIN	CDNA EMBL ACC#	SPLICE VARIANTS (PROTEIN)	MUTATIONS	GENE EMBL ACC#
Glutamate gamma carboxylase	BC013979	2; BC013979; AF253530	1 SNP (EMBL# U65896); 2 SNPs (OMIM# 137167)	U65896
Prothrombin	V00595	1; V00595	approx. 100 SNP's (EMBL# AF478696)	AF478696

Each of these proteins, including their nucleic acid and amino acid sequences, are well known. Table 2 identifies representative sequences of wild-type and mutant forms of the various proteins that can be used in the present invention.

The term “gamma-glutamyl carboxylase” or “GGCX”, as used herein, refers to a vitamin K dependent enzyme that catalyses carboxylation of glutamic acid residues.

GGCX enzymes are widely distributed, and have been cloned from many different species such as the beluga whale *Delphinapterus leucas*, the toadfish *Opsanus tau*, chicken (*Gallus gallus*), hagfish (*Myxine glutinosa*), horseshoe crab (*Limulus polyphemus*), and the cone snail *Conus textile* (Begley et al., 2000, *ibid*; Bandyopadhyay et al. 2002, *ibid*). The carboxylase from conus snail is similar to bovine carboxylase and has been expressed in

COS cells (Czerwiec et al. 2002, *ibid*). Additional proteins similar to GGCX can be found in insects and prokaryotes such as *Anopheles gambiae, Drosophila melanogaster* and *Leptospira* with NCBI accession numbers: gi 31217234, gi 21298685, gi 24216281, gi 24197548 and (Bandyopadhyay et al., 2002, *ibid*), respectively. The carboxylase enzyme displays remarkable evolutionary conservation. Several of the non-human enzymes have shown, or may be predicted to have, activity similar to that of the human GGCX we have used, and may therefore be used as an alternative to the human enzyme.

Table 2 identifies representative sequences of predicted proteins homologous to human GGXC (sorted after species origin) that can be used in the present invention.

Table 2

Species	**Data base accession #/ID**
Homo sapiens (man)	NM_000821.2 HUMGLUCARB HUMHGCA BC004422 HSU65896 AF253530.1
Papio hamadryas (red baboon)	AC116665.1
Delphinapterus leucas (white whale)	AF278713
Bos taurus (bovine)	NM_174066.2 BOVCARBOXG BOVBGCA
Ovis aries (domestic sheep)	AF312035
Rattus norvegicus (brown rat)	NM_031756.1 AF065387
Mus musculus (mouse)	NM_019802.1 AF087938
Opsanus tau (bony fishes)	AF278714.1
Conus textile (molluscs)	AY0044904.1

	AF382823.2
Conus imperialis (molluscs)	AF448234.1
Conus episcopatus (molluscs)	AF448233.1
Conus omaria (molluscs)	AF448235.1
Drosophila melanogaster (fruit fly)	NM_079161.2
Anopheles gambiae (mosquito)	XM_316389.1
Secale cereale (monocots)	SCE314767
Triticum aestivum (common wheat)	AF280606.1
Triticum urartu (monocots)	AY245579.1
Hordeum vulgare (barley)	BLYHORDCA
Leptospira interrogans (spirochetes)	AE011514.1
Streptomyces coelicolor (high GC Gram+ bacteria)	SCO939109 SCO939124 AF425987.1
Streptomyces lividans (high GC Gram+ bacteria)	SLU22894
Streptomyces viginiae (high GC Gram+ bacteria)	SVSNBDE
Micrococcus luteus (high GC Gram+ bacteria)	MLSPCOPER
Chlamydomonas reinhardtii (green algae)	AF479588.1
Dictyostelium discoideum (slime mold)	AC115612.2
Coturnix coturnix (birds)	AF364329.1
Bradyrhizobium japonicum (α-protoebacteria)	AP005937.1
Rhodobacter sphaeroides (α-proteobacteria)	RSY14197
Sinorhizobium meliloti (α-proteobacteria)	RME603647 AF119834
Mesorhizobium loti (α-proteobacteria)	AP003014.2
Chromobacterium violaceum (β-proteobacteria)	AE016910.1 AE016918.1
Pseudomonas aeruginosa (γ-proteobacteria)	AE004613.1 AF165882
Xanthomonas axonopodis (γ-proteobacteria)	AE011706.1
Human herpesvirus 8	KSU52064

	KSU75698 AF305694 AF360120 AF192756

Each of the above-identified GGCX proteins and GGCX proteins from other species can be used as the carboxylase enzyme in the present invention.

A second part of the invention is a cell line stably transfected with a polynucleotide encoding ecarin and associated control elements (Figure 2). The ecarin encoding sequence may be optimised for expression in mammalian cells, but is not limited to such sequences. In one embodiment of the invention the sequence according to SEQ ID NO 2 or a homologue thereof is used to express ecarin. A homologue of SEQ ID NO 2 coding for ecarin may have at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identity to the sequence SEQ ID NO 2. The host cell is preferably a eukaryotic cell. Typical host cells include, but are not limited to insect cells, yeast cells, and mammalian cells. Mammalian cells are particularly preferred. Suitable mammalian cells lines include, but are not limited to, CHO, HEK, NS0, 293, Per C.6, BHK and COS cells, and derivatives thereof. In one embodiment the host cell is the mammalian cell line CHO-S.

In one embodiment prothrombin and ecarin are produced from cells originating from the same parent cell line. This cell line origin may be, but is not limited to, Chinese Hamster Ovary cells (CHO) including derivatives and NS0 (myeloma BALB/c mouse) including derivatives. The purpose of using the same cell line background is to facilitate purification and evaluation of purity of the thrombin product.

In another embodiment ecarin and prothrombin are produced from different host cell line; i.e. CHO and NS0, respectively.

In one aspect of the invention use of recombinant ecarin is preferred as this facilitates detection of non-thrombin product derived components during the thrombin generation process and in the final thrombin product. In a second aspect recombinant ecarin is preferred due to reduced risk for exposure to allergenic or toxic components that may be present in ecarin derived from snake venom. In a third aspect ecarin from snake venom is not preferred due to batch variation and limited batch size of ecarin preparations.

The crude prothrombin and the crude ecarin are mixed and incubated under conditions that allow formation of thrombin, such as described in Example 3. Generated thrombin is then purified by methods described in Example 4 or by other methods known by persons skilled in the art. Alternatively prothrombin and/or ecarin can first be purified by methods known in the art and then mixed to obtain thrombin. Thrombin is then purified from non-product components.

An example of a suitable thrombin manufacturing process is outlined in Figure 3.

A method is provided for producing recombinant human thrombin from recombinant prothrombin using recombinant ecarin having the sequence SEQ ID NO 2 or a homologue thereof. The recombinant ecarin can be expressed and secreted by a cell containing the gene comprising the nucleotide sequence SEQ ID NO 2 or a homologue thereof in CHO-S cells, which ecarin has an amino acid sequence equal to that of wild type ecarin.

In the above method the recombinant protrombin is subjected to recombinant ecarin, which recombinant ecarin can be isolated in active form after extra-cellular expression by CHO-S cells, said cells being left to apoptosis/necrosis for a time sufficient to activate said ecarin, whereupon a human recombinant thrombin is isolated.

The recombinant prothrombin can be produced by a cell-line comprising a prothrombin expressing gene having a nucleotide sequence comprising the sequence SEQ. ID. NO. 1 or an homologue thereof. A homologue of SEQ ID NO 1 coding for prothrombin may have at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identity to the sequence SEQ ID NO 1. The recombinant prothrombin can be a mixture of fully carboxylated prothrombin and incompletely carboxylated prothrombin. In one embodiment, the recombinant prothrombin is a fully carboxylated prothrombin and in another embodiment, the recombinant prothrombin is an incompletely carboxylated prothrombin.

A further aspect of the invention relates to the recombinant thrombin obtained by the method according to the invention. A pharmaceutical composition can be designed comprising the recombinant thrombin obtained be the method according to the invention, in combination with pharmaceutically acceptable carriers, vehicles and/or adjuvants.The pharmaceutical composition can be in an applicable form.

In one embodiment thrombin produced by the described method can be used in the manufacturing of tissue sealants ("glues") in combination with other proteins, i.e. fibrin originating from recombinant cells, transgenic animals or human plasma. In another embodiment thrombin produced by the described method can be used as a stand-alone product, freeze dried as single active component or in combination with a non-protein matrix, or, in solution as single active component or in combination with other active components.

Suitable mix-in components would be, but is not limited to, collagen, chitin, degradable polymers, cellulose, recombinant coagulation factors and fibrinogen from transgenic or recombinant sources.

The potential fields of use for the tissue sealants ("glues") are numerous; skin grafting, neuro surgery, cardiac surgery, toracic surgery, vascular surgery, oncologic surgery, plastic surgery, ophthalmologic surgery, orthopedic surgery, trauma surgery, head and neck surgery, gynecologic and urologic surgery, gastrointestinal surgery, dental surgery, drug delivery, tissue engineering and dental cavity haemostasis.

A further aspect of the invention relates to a method for obtaining coagulation by administering a therapeutically effective amount of a recombinant human thrombin obtained using the method according to the invention to a patient.

Another aspect of the present invention is an isolated DNA sequence according SEQ ID NO 2 or homologues therof coding for a recombinant ecarin. A homologue of SEQ ID NO 2 coding for ecarin may have at least 80%, 85%, 90%, 95%, 97%, 98% or 99% identity to the sequence SEQ ID NO 2. SEQ ID NO 2 is a designed sequence that has been optimised for optimal expression. The sequence is particularly suited for expression in mammalian cell systems.

According to another aspect a vector comprising SEQ ID NO 2 or a homologue thereof is provided. Said vector can be designed to overexpress SEQ ID NO 2 or a homologue thereof and is operably linked to expression control sequences permitting expression of ecarin encoded by SEQ ID NO 2 or a homologue thereof. According to a third aspect a host cell comprising said vector is provided that is capable of expressing ecarin encoded by SEQ ID NO 2 or a homologue thereof. This host cell is preferably a eukaryotic cell. Typical host cells include, but are not limited to insect cells, yeast cells, and mammalian cells. Mammalian cells are particularly preferred. Suitable mammalian cells lines include, but are not limited to, CHO, HEK, NS0, 293, Per C.6, BHK and COS cells, and derivatives thereof. In one embodiment the host cell is the mammalian cell line CHO-S.

According to another embodiment of the present invention a polypeptide comprising an amino acid sequence encoded by SEQ ID NO: 2 or a homologue thereof and obtained by the method described in Example 2.

The sequence identity between two sequences can be determined by pair-wise computer alignment analysis, using programs such as, BestFit, PILEUP, Gap or FrameAlign. The preferred alignment tool is BestFit. In practise, when searching for similar/identical sequences to the query search, from within a sequence database, it is generally necessary to perform an initial identification of similar sequences using suitable algorithms such as Blast, Blast2, NCBI Blast2, WashU Blast2, FastA, or Fasta3, and a scoring matrix such as Blosum 62. Such algorithms endeavour to closely approximate the "gold-standard" alignment algorithm of Smith-Waterman. Thus, the preferred software/search engine program for use in assessing similarity, i.e., how two primary polypeptide sequences line up is Smith-Waterman. Identity refers to direct matches, similarity allows for conservative substitutions.

Experimental section

The invention will be further described by means of the following examples which shall not be interpreted as limiting the scope of the appended claims.

Example 1

High yield production of recombinant human prothrombin in CHO cells.

The P1E2 cell line containing the construct PN32 shown in Figure 1 having the nucleotide sequence SEQ ID NO: 1, was grown in a fermentor according to the method described in WO2005038019, using a protein and animal component free growth medium in order to produce prothrombin for use in thrombin manufacturing. The cells were grown either by batch or perfusion culture methods (Table 1) and the amount of prothrombin produced was measured by an ecarin assay. This ecarin assay was performed essentially as the Chromogenix assay (Mölndal, Sweden) using purified plasma-derived human prothrombin (Haematologic Technologies Inc., Vermont, USA) as standard.

Table 1. Examples of yield of prothrombin in experimental fermentor runs

Experiment ID	Culture method & time	Viable cells (million cells/mL)	Prothrombin mg/L
CC2LC (272-8)	Batch, 238 h	5.9	281
CC2LD (272-8)	Batch, 238 h	6.2	276
326-11B	Perfusion, 259 h	18	722

The fermentor experiments showed that both batch and perfusion culture methods can be used to produce prothrombin suitable for production of recombinant thrombin (Table 1). The share of fully carboxylated prothrombin obtained in these fermentor runs was about 55-87 %, the rest being incompletely carboxylated prothrombin.

Example 2

Production of recombinant ecarin in CHO cells.

An ecarin encoding sequence having the nucleotide sequence SEQ ID NO: 2 optimised for expression in mammalian cells was synthesized and cloned into the Invitrogen vector pCDNA 3.1+ (Figure 2). An alignment of the nucleotide sequence of the recombinant ecarin used in the present invention to the sequence of wild type ecarin (GI:717090) is seen in Figure 4. As can be seen in Figure 5 this recombinant ecarin is 100 % homologous to the amino acid sequence for wild type ecarin. This construct, AZ ecarin (SEQ ID NO. 3), was used to stably transfect CHO-S cells (Invitrogen). Ecarin is secreted by the host cell to the extra-cellular space, and in order to screen for ecarin producing clones, culture supernatant samples were removed and mixed with recombinant human prothrombin (rhFII) to a final concentration of 1 mg rhFII /L in assay buffer (50 mM Tris-HCl, pH 7.4 containing 0.1% BSA). This mix was incubated 20-40 minutes at 37°C. The thrombin generated by the action of ecarin present in the sample was then detected by adding a 1-2 mM solution of the chromogenic thrombin substrate S-2238 (Chromogenix, Mölndal). Colour development was monitored and stopped when suitable using 20% acetic acid. To estimate the activity of the recombinant ecarin produced, snake venom derived ecarin with a declared activity was purchased from Sigma and used as standard. The best producing cell line obtained produced up to 7000 U ecarin per litre culture in lab scale shaker cultures grown in animal component free medium.

Activation of recombinant ecarin

The above method produces the recombinant ecarin as a pro protein Thus, activation by removal of the pro-part is necessary for optimal activity. To our surprise, we found that activation was most conveniently obtained by continued incubation of the culture for at least 7 days after the death of the ecarin producing cells (Figure 6). The culture medium used was CD-CHO supplemented with HT-supplement, non-essential amino acids and Glutamax I (as recommended by Invitrogen for CHO-S), and growth conditions were shaker bottles at 37°C in an atmosphere containing 5% carbon dioxide. Culture samples were assayed for activity as described above. As can be seen from Figure 6, the activity of recombinant ecarin increased during the activation period.

Samples from culture supernatants were also separated by SDS-PAGE and blotted to nitrocellulose mebranes. Labelling of the membrane was performed with polyclonal rabbit serum directed towards the mature part of ecarin expressed as inclusion bodies in *E. coli*. "M" indicates the molecular weight marker and numbers refer to day of sample collection. As can be seen from Figure 7 the recombinant ecarin remains stable for more than a week after the death of the cells. Activation of ecarin may also take place at lower temperatures, for instance as low as room temperature, but will then require longer times for activation. Ecarin will remain stable for severable months in room temperature in the presence of dead host cells. The activity will increase gradually until it levels out. A decrease in activity has not been observed except in the presence of bacterial infections or high temperatures. Efforts to use trypsin for activation of ecarin were made, but were not successful.

Example 3

Conversion of prothrombin to thrombin by ecarin.

The ecarin protease converts prothrombin to meizothrombin, an intermediate form of thrombin that has thrombin catalytic activity. Further processing into thrombin is achieved by auto-catalyses. To determine the estimated amount of ecarin culture needed for converting prothrombin into thrombin, we performed a series of test digests. Different amounts of ecarin-containing culture supernatants as obtained in Example 2, were mixed with 1 mg/ml prothrombin (as obtained in example 1) in PBS buffer (Cambrex). Incubation of the mixtures was done at 37°C for 1-3 hours. Samples were then analysed by SDS-PAGE to identify the amount of recombinant ecarin needed for complete conversion of prothrombin into thrombin. By this procedure we found that the recombinant ecarin was very potent; one litre of ecarin culture supernatant at 7000 U/L is capable of complete conversion of 64 grams of prothrombin into thrombin in less than 3 hours at 37°C. Normally recombinantly produced prothrombin has to be purified in order to separate fully-carboxylated prothrombin from the incompletely carboxylated prothrombin. However this is not necessary for the present invention as the recombinant ecarin is able to efficiently activate both the fully carboxylated and the incompletely carboxylated prothrombins.

Example 4

Purification of thrombin

Thrombin obtained by the procedure described in example 3 was purified by cation-exchange chromatography (CIEX) using ÄKTA-FPLC (GE Healthcare) and an SP-Sepharose HP column (GE Healthcare) equilibrated with 25 mM sodium-phosphate buffer, pH 6.5. Ecarin-digested prothrombin prepared as in example 3 was adjusted to pH 6.2 and a conductivity of approximately 8 mS/cm. Thrombin was eluted with a 1M sodium chloride gradient in column equilibration buffer over 20 column volumes (Figure 8). Selected fractions were analysed by SDS-PAGE (Figure 9). Thrombin activity was confirmed by incubation with the chromogenic thrombin substrate S-2238 (Chromogenix, Mölndal).

Example 5

Analyses of rh-thrombin obtained.

To further analyse the obtained thrombin, kinetic parameters were determined using the chromogenic thrombin substrate S-2366 (Chromogenix). Activity was estimated by titration with hirudin. The rh-thrombin was for all parameters; Activity, K_{kat} and V_{max}, similar to plasma-derived human α-thrombin from Haematologic Technologies Inc. (Vermont, USA).

Purified thrombin was also subjected to N-terminal sequencing: Reduced thrombin heavy and light polypeptide chains were separated by SDS-PAGE and blotted to Immobilon P membrane (Millipor). The excised bands were sequenced by the Edman degradation method. Heavy chain N-terminal first five amino acids were confirmed to be IVEGS, and the light chain five N-terminal amino acids were TFGS as expected.

2008275828 25 May 2012

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method comprising:

(a) providing mammalian cells comprising DNA encoding prepro-ecarin comprising SEQ ID NO: 5;

(b) expressing the prepro-ecarin in the cells by incubating the cells under conditions permitting the cells to express the prepro-ecarin from the DNA;

(c) continuing to incubate the cells until substantially all of the cells have ceased to be viable, thereby producing a medium comprising dead cells; and

(d) continuing to incubate the medium for an activation period sufficient to produce active ecarin from the prepro-ecarin in the medium.

2. The method of claim 1, wherein the activation period of (d) lasts at least seven days after substantially all cells have ceased to be viable.

3. The method of claim 1 or claim 2, further comprising fractionating the active ecarin-containing medium of (d) to separate a fraction containing active ecarin from a fraction containing dead cells.

4. The method of any one of claims 1 to 3, wherein the cells are stably transfected with the DNA encoding prepro-ecarin.

5. The method of any one of claims 1 to 4, wherein the DNA encoding prepro-ecarin comprises the nucleotide sequence of SEQ ID NO 2.

6. The method of any one of claims 1 to 5, wherein the cells are CHO-S cells.

7. The method of claim 6, wherein the CHO-S cells are stably transfected with a DNA comprising the nucleic acid sequence of SEQ ID NO 2.

8. The method of claim 1, further comprising:

2008275828 25 May 2012

providing recombinant human prothrombin; and

contacting a sample of the active ecarin-containing medium with the recombinant human prothrombin, thereby producing recombinant human thrombin.

9. The method of claim 8, wherein the recombinant human prothrombin and the prepro-ecarin are expressed using cultured mammalian cells obtained from the same parental cell line.

10. The method of claim 9, wherein the parental cell line is CHO-S.

11. The method of claim 10, wherein the cells of (a) are CHO-S cells stably transfected with a DNA comprising the nucleic acid sequence of SEQ ID NO 2.

12. The method of claim 8, wherein the recombinant human prothrombin is a mixture of fully carboxylated prothrombin and incompletely carboxylated prothrombin.

13. The method of any one of claims 8 to 12, wherein the recombinant prothrombin is fully carboxylated.

14. The method of any one of claims 8 to 12, wherein the recombinant prothrombin is incompletely carboxylated.

15. The method of any one of claims 8 to 14, further comprising purifying the recombinant human thrombin.

16. The method of claim 15, further comprising formulating the purified recombinant human thrombin in a pharmaceutical composition.

17. The method of claim 9, wherein the cultured mammalian cells expressing recombinant human prothrombin also comprise recombinant DNA encoding a gamma-glutamyl carboxylase and express recombinant gamma-glutamyl carboxylase.

18. The method of claim 17, wherein the level of expression of prothrombin by the cells expressing recombinant human prothrombin is at least ten times the level of expression of gamma-glutamyl carboxylase.

19. The method of claim 17, wherein the mammalian cells expressing recombinant human prothrombin express the recombinant prothrombin and recombinant gamma-glutamyl carboxylase while being cultured in a medium to which no vitamin K has been added.

20. The method of claim 17, wherein the recombinant prothrombin expressed in the cells expressing recombinant human prothrombin is incompletely carboxylated prothrombin or a mixture of fully and incompletely carboxylated prothrombin.

21. The method of claim 18, wherein the recombinant prothrombin expressed in the cells expressing recombinant human prothrombin is incompletely carboxylated prothrombin or a mixture of fully and incompletely carboxylated prothrombin.

22. The method of claim 19, wherein the recombinant prothrombin expressed in the cells expressing recombinant human prothrombin is incompletely carboxylated prothrombin or a mixture of fully and incompletely carboxylated prothrombin.

23. The method of claim 9, wherein the mammalian cells expressing recombinant human prothrombin express the recombinant prothrombin while being cultured in a medium to which no vitamin K has been added.

24. The method of claim 1, further comprising

producing, from the medium comprising dead cells, a culture supernatant comprising active ecarin; and

contacting prothrombin with the culture supernatant, thereby producing thrombin.

25. The method of claim 24, wherein the prothrombin is incompletely carboxylated prothrombin.

26. The method of claim 24, wherein the prothrombin is a mixture of fully carboxylated prothrombin and incompletely carboxylated prothrombin.

27. The method of claim 1, further comprising producing, from the medium of (d), a culture supernatant comprising active ecarin.

28. The method of claim 27, further comprising

providing recombinant prothrombin; and

contacting part or all of the culture supernatant with the recombinant prothrombin, thereby producing recombinant thrombin.

29. The method of claim 1, further comprising

providing recombinant prothrombin; and

contacting the active ecarin with the recombinant prothrombin, thereby producing recombinant thrombin,

wherein the active ecarin is not purified from the medium prior to being contacted with the recombinant prothrombin.

30. The method of claim 27, further comprising

providing recombinant prothrombin; and

contacting the active ecarin with the recombinant prothrombin, thereby producing recombinant thrombin,

wherein the active ecarin is not purified from the culture supernatant prior to being contacted with the recombinant prothrombin.

31. The method of claim 1, wherein the DNA encoding prepro-ecarin comprises a nucleotide sequence at least 85% identical to SEQ ID NO 2.

32. The method of claim 1, wherein the DNA encoding prepro-ecarin comprises a nucleotide sequence at least 90% identical to SEQ ID NO 2.

33. The method of claim 1, wherein the DNA encoding prepro-ecarin comprises a nucleotide sequence at least 95% identical to SEQ ID NO 2.

34. The method of claim 1, wherein the DNA encoding prepro-ecarin comprises a nucleotide sequence at least 97% identical to SEQ ID NO 2.

35. The method of claim 1, wherein the DNA encoding prepro-ecarin comprises a nucleotide sequence at least 98% identical to SEQ ID NO 2.

36. The method of claim 1, wherein the DNA encoding prepro-ecarin comprises a nucleotide sequence at least 99% identical to SEQ ID NO 2.

37. A method of claim 1 substantially as hereinbefore described with reference to any one of the examples.

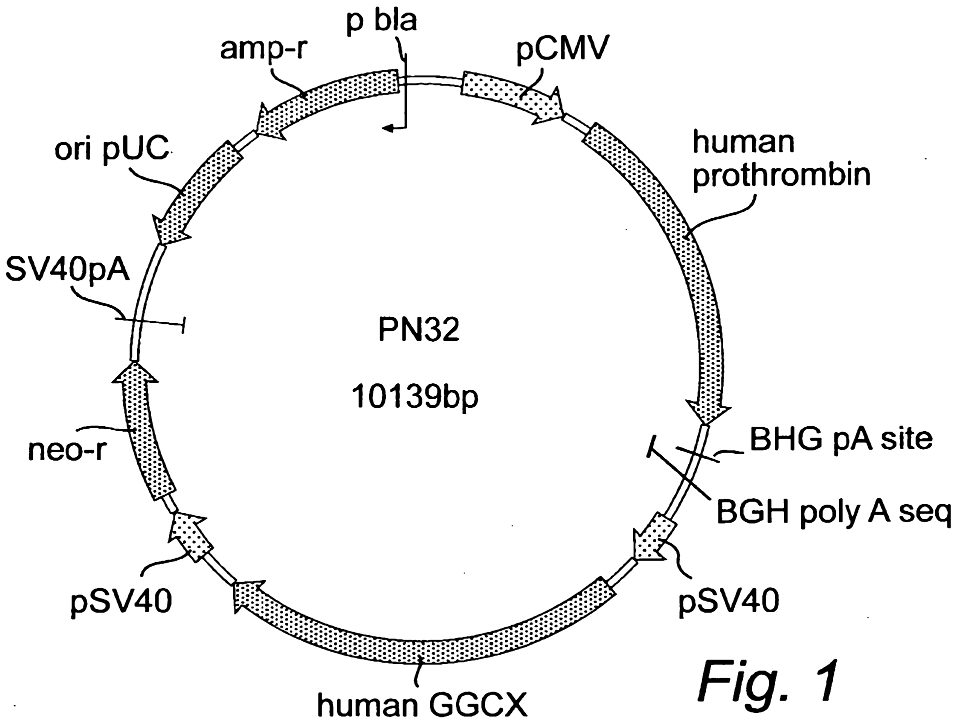

p bla
amp-r
pCMV
ori pUC
human prothrombin
SV40pA
PN32
10139bp
neo-r
BHG pA site
BGH poly A seq
pSV40
pSV40
human GGCX

Fig. 1

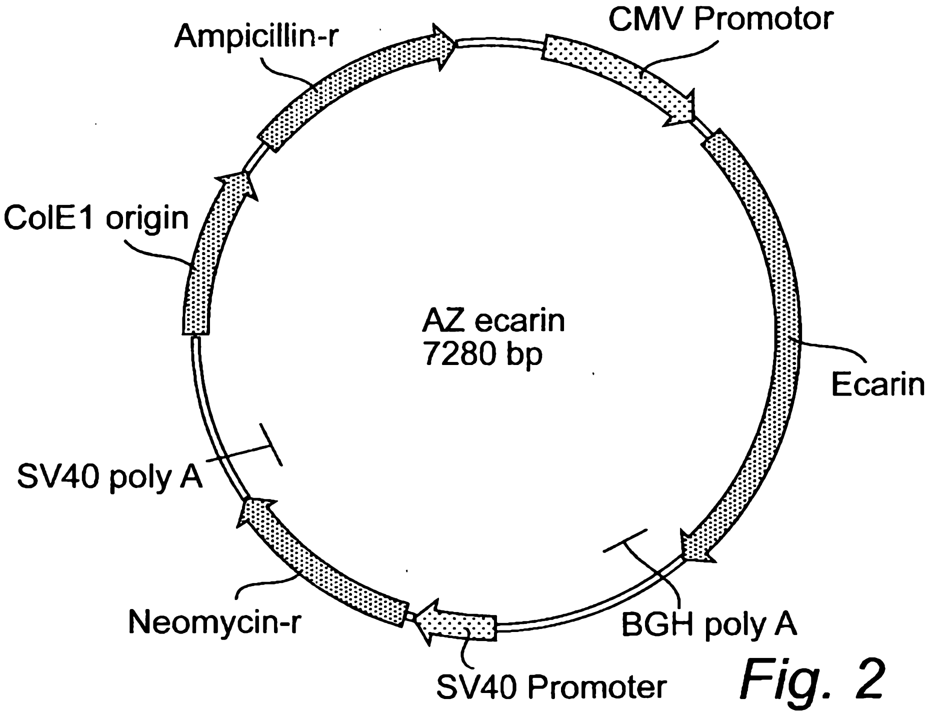

Ampicillin-r
CMV Promotor
ColE1 origin
AZ ecarin
7280 bp
Ecarin
SV40 poly A
Neomycin-r
BGH poly A
SV40 Promoter

Fig. 2

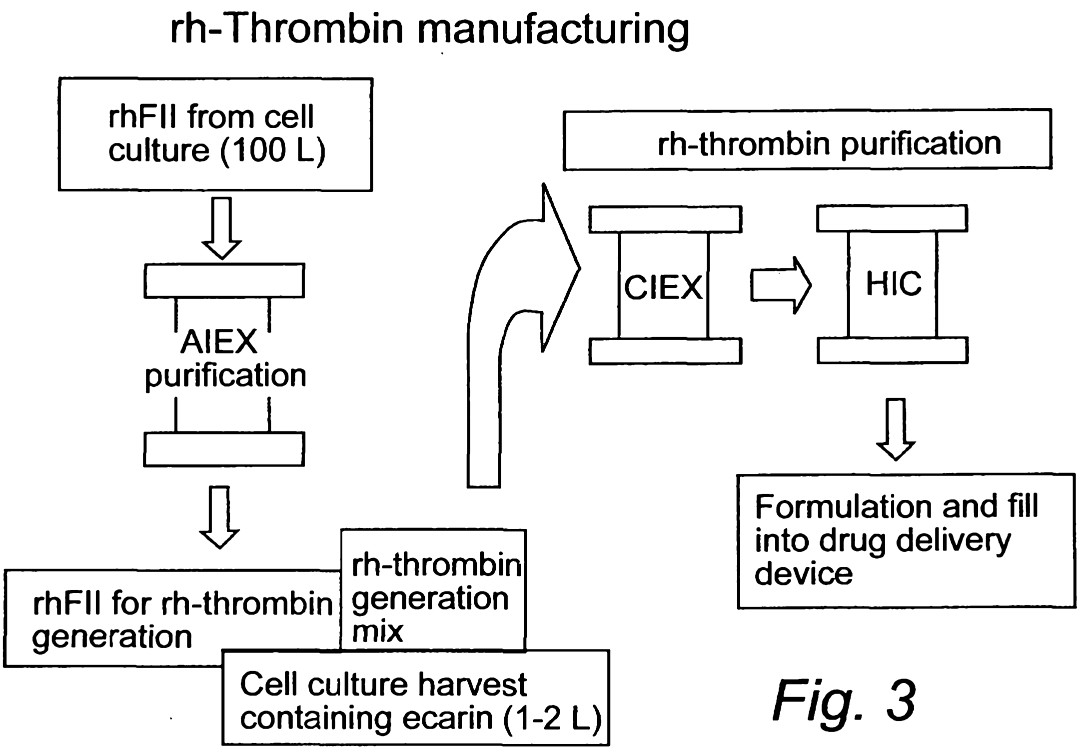

rh-Thrombin manufacturing
rhFII from cell culture (100 L)
AIEX purification
rhFII for rh-thrombin generation
rh-thrombin generation mix
Cell culture harvest containing ecarin (1-2 L)
rh-thrombin purification
CIEX
HIC
Formulation and fill into drug delivery device

Fig. 3

Fig. 4

```
Score =  744 bits (387),  Expect = 0.0
Identities = 1365/1848 (73%), Gaps = 0/1848 (0%)
Strand=Plus/Plus

AZ ecarin  ATGATCCAGATCCTGCTGGTGATCATCTGCCTGGCCGTGTTCCCCTACCAGGGCTGCTCC  60
WT ecarin  ATGATCCAGATTCTCTTGGTAATTATATGCTTAGCAGTTTTCCATATCAAGGTTGCTCT  60

AZ ecarin  ATCATCCTGGGCAGCGGCAACGTGAACGACTACGAGGTGGTGTACCCCCAGAAGGTGACC  120
WT ecarin  ATAATCCTGGGATCTGGGAATGTTAATGATTATGAAGTAGTGTATCCACAAAAAGTCACT  120

AZ ecarin  GCCCTGCCCAAGGGCGCCGTGCAGCAGCCCGAGCAGAAATACGAGGACGCCATGCAGTAC  180
WT ecarin  GCATTGCCCAAAGGAGCAGTTCAGCAGCCTGAGCAAAAGTATGAAGATGCCATGCAATAT  180

AZ ecarin  GAGTTCGAGGTGAAGGGCGAGCCCGTGGTGCTGCACCTGGAGAAGAACAAGGAGCTGTTC  240
WT ecarin  GAATTTGAAGTGAAGGGAGAGCCAGTGGTCCTTCACCTAGAAAAAAATAAAGAACTTTTT  240

AZ ecarin  AGCGAGGACTACAGCGAGACCCACTACAGCAGCGACGACAGGGAGATCACCACCAACCCC  300
WT ecarin  TCAGAAGATTACAGTGAGACTCATTATTCGTCTGATGACAGAGAAATTACAACAAACCCT  300

AZ ecarin  AGCGTGGAGGACCACTGCTACTACCACGGCCGGATCCAGAACGACGCCGAGAGCACCGCC  360
WT ecarin  TCAGTTGAGGATCACTGCTATTATCATGGACGGATCCAGAATGATGCTGAGTCAACTGCA  360

AZ ecarin  AGCATCAGCGCCTGTAATGGCCTGAAGGGCCACTTCAAGCTGAGAGGCGAGACCTACTTC  420
WT ecarin  AGCATCAGTGCATGCAATGGTTTGAAAGGACATTTCAAGCTTCGAGGGGAGACGTACTTT  420
```

```
AZ ecarin  ATCGAGCCCCTGAAGATCCCCGACAGCGAGGCCCACGCCGTGTACAAGTACGAGAACATC  480
WT ecarin  ATTGAACCCTTGAAGATTCCCGACAGTGAAGCCCATGCAGTCTACAAATATGAAAACATA  480

AZ ecarin  GAGAACGAGGACGAGGCCCCTAAGATGTGTGGCGTGACCCAGGACAACTGGGAGAGCGAC  540
WT ecarin  GAAAATGAGGATGAAGCCCCCAAAATGTGTGGGGTAACCCAGGATAATTGGGAATCAGAT  540

AZ ecarin  GAGCCCATCAAGAAAACCCTGGGCCTGATCGTGnnnnnnnACGAGAGAAAGTTCGAGAAG  600
WT ecarin  GAACCCATCAAAAAGACTTTGGGGTTAATTGTTCCTCCTCATGAACGAAAATTTGAGAAA  600

AZ ecarin  AAGTTCATCGAACTGGTGGTCGTGGTGGACCACAGCATGGTGACCAAGTACAACAACGAC  660
WT ecarin  AAATTCATTGAGCTTGTCGTAGTTGTGGACCACAGTATGGTCACAAAATACAACAATGAT  660

AZ ecarin  ACCACCCCCATCACCACCTCCATCTACCACATGCTCAACACCCTCAACCACATCTACCTC  720
WT ecarin  TCAACTGCTATAAGAACATGGATATATGAAATGCTCAACACTGTAAATGAGATATACTTA  720

AZ ecarin  CCCTTCAACATCAGAGTGGCCCTGGTGGGCCTGGAGTTCTGGTGTAACGGCGACCTGATC  780
WT ecarin  CCTTTCAATATTCCTCTACCACTCCTTCCCCTACAATTTTCCTCCAATCCACACTTCATT  780

AZ ecarin  AACGTGACCAGCACCGCCGACGACACCCTGCACAGCTTCGGCGAGTGGAGAGCCAGCGAC  840
WT ecarin  AACGTGACATCCACAGCAGATGATACTTTGCACTCATTTGGAGAATGGAGAGCATCAGAT  840
```

Fig. 4

```
AZ ecarin  CTGCTGAACCGGAAGAGACACGATCACGCCCAGCTGCTGACCAATGTGACCCTGGACCAC  900
WT ecarin  TTGCTGAATCGAAAAAGACATGATCATGCTCAGTTACTCACGAACGTGACACTGGATCAT  900

AZ ecarin  TCCACCCTGGGCATCACCTTCGTGTACGGCATGTGTAAGAGCGACCGGAGCGTGGAGCTG  960
WT ecarin  TCCACTCTTGGAATCACGTTCGTATATGGCATGTGCAAATCAGATCGTTCTGTAGAACTT  960

AZ ecarin  ATCCTGGACTACAGCAACATCACCTTCAACATGGCCTACATCATCGCCCACGAGATGGGC  1020
WT ecarin  ATTCTGGATTACAGCAACATAACTTTTAATATGGCATATATAATAGCCCATGAGATGGGT  1020

AZ ecarin  CACAGCCTGGGCATGCTGCACGACACCAAGTTCTGTACCTGTGGCGCCAAGCCCTGTATC  1080
WT ecarin  CATAGTCTGGGCATGTTACATGACACAAAATTCTGTACTTGTGGGGCTAAACCATGCATT  1080

AZ ecarin  ATGTTCGGCAAGGAGAGCATCCCTCCCCCTAAGGAGTTCAGCAGCTGCTCCTACGACCAG  1140
WT ecarin  ATGTTTGGCAAAGAAAGCATTCCACCGCCCAAAGAATTCAGCAGTTGTAGTTATGACCAG  1140

AZ ecarin  TACAATAAGTACCTGCTGAAGTACAACCCCAAGTGTATCCTGGAnnnnnnnnTGAGAAAG  1200
WT ecarin  TATAACAAGTATCTTCTTAAATATAACCCAAAATGCATTCTTGATCCACCTTTGAGAAAA  1200
```

Fig. 4

```
AZ ecarin  GACATCGCCAGCCCTGCCGTGTGTGGCAATGAGATCTGGGAGGAGGGCGAGGAGTGTGAC  1260
WT ecarin  GATATTGCTTCACCTGCAGTTTGTGGAAATGAAATTTGGGAGGAAGGAGAAGAATGTGAT  1260

AZ ecarin  TGTGGCAGCCCAGCCGACTGTAGAAACCCCTGCTGTGATGCCGCCACCTGTAAGCTGAAG  1320
WT ecarin  TGTGGTTCTCCTGCAGATTGTCGAAATCCATGCTGTGATGCTGCAACATGTAAACTGAAA  1320

AZ ecarin  CCTGGCGCCGAGTGTGGCAACGGCGAGTGCTGTGACAAGTGTAAGATCCGGAAGGCCGGC  1380
WT ecarin  CCAGGGGCAGAATGTGGAAATGGAGAGTGTTGTGACAAGTGCAAGATTAGGAAAGCAGGA  1380

AZ ecarin  ACCGAGTGTAGACCCGCCAGGGACGATTGTGACGTGGCCGAGCACTGTACCGGCCAGAGC  1440
WT ecarin  ACAGAATGCCGGCCAGCAAGGGATGACTGTGATGTCGCTGAACACTGCACTGGCCAATCT  1440

AZ ecarin  GCCGAGTGCCCCAGAAACGAGTTCCAGAGGAACGGCCAGCCTTGCCTGAACAACAGCGGC  1500
WT ecarin  GCTGAGTGTCCCAGAAATGAGTTCCAAAGGAATGGACAACCATGCCTTAACAACTCGGGT  1500

AZ ecarin  TACTGCTACAACGGCGACTGCCCCATCATGCTGAACCAGTGTATCGCCCTGTTCAGCCCC  1560
WT ecarin  TATTGCTACAATGGGGATTGCCCCATCATGTTAAACCAATGTATTGCTCTCTTTAGTCCA  1560

AZ ecarin  AGCGCCACCGTGGCCCAGGACAGCTGCTTCCAGAGAAACCTGCAGGGCAGCTACTACGGC  1620
WT ecarin  AGTGCAACTGTGGCTCAAGATTCATGTTTTCAGAGGAACTTGCAAGGCAGTTACTATGGC  1620
```

Fig. 4

```
AZ ecarin   TACTGTACCAAGGAGATCGGCTACTACGGAAAGAGGTTCCCCTGTGCCCCTCAGGACGTG   1680
WT ecarin   TACTGCACAAAGGAAATTGGTTACTATGGTAAAAGGTTTCCATGTGCACCACAAGATGTA   1680

AZ ecarin   AAGTGTGGCAGGCTGTACTGCCTGGACAACTCCTTCAAGAAAAACATGAGGTGTAAGAAC   1740
WT ecarin   AAATGTGGCAGATTATACTGCTTAGATAATTCATTCAAAAAAAATATGCGTTGCAAGAAC   1740

AZ ecarin   GACTACAGCTACGCCGACGAGAACAAGGGCATCGTGGAGCCCGGCACCAAGTGTGAGGAC   1800
WT ecarin   GACTATTCATACGCGGATGAAAATAAGGGAATAGTTGAACCTGGAACAAAATGTGAAGAT   1800

AZ ecarin   GGCAAAGTGTGTATCAACCGGAAGTGTGTGGACGTGAACACCGCCTAC   1848
WT ecarin   GGAAAGGTCTGCATCAACAGGAAGTGTGTTGATGTGAATACAGCCTAC   1848
```

Fig. 4

Fig. 5

```
gi|217090|dbj|D32212.1|EHCEC  Echis carinatus mRNA for ecarin
Length=2349

 Score = 1315 bits (3404),  Expect = 0.0
 Identities = 616/616 (100%), Positives = 616/616 (100%), Gaps = 0/616 (0%)
 Frame = +3

AZ ecarin  1     MIQILLVIICLAVFPYQGCSIILGSGNVNDYEVVYPQKVTALPKGAVQQPEQKYEDAMQY  60
WT ecarin        MIQILLVIICLAVFPYQGCSIILGSGNVNDYEVVYPQKVTALPKGAVQQPEQKYEDAMQY
           111   MIQILLVIICLAVFPYQGCSIILGSGNVNDYEVVYPQKVTALPKGAVQQPEQKYEDAMQY  290

AZ ecarin  61    EFEVKGEPVVLHLEKNKELFSEDYSETHYSSDDREITTNPSVEDHCYYHGRIQNDAESTA  120
WT ecarin        EFEVKGEPVVLHLEKNKELFSEDYSETHYSSDDREITTNPSVEDHCYYHGRIQNDAESTA
           291   EFEVKGEPVVLHLEKNKELFSEDYSETHYSSDDREITTNPSVEDHCYYHGRIQNDAESTA  470

AZ ecarin  121   SISACNGLKGHFKLRGETYFIEPLKIPDSEAHAVYKYENIENEDEAPKMCGVTQDNWESD  180
WT ecarin        SISACNGLKGHFKLRGETYFIEPLKIPDSEAHAVYKYENIENEDEAPKMCGVTQDNWESD
           471   SISACNGLKGHFKLRGETYFIEPLKIPDSEAHAVYKYENIENEDEAPKMCGVTQDNWESD  650

AZ ecarin  181   EPIKKTLGLIVPPHERKFEKKFIELVVVVDHSMVTKYNNDSTAIRTWIYEMLNTVNEIYL  240
WT ecarin        EPIKKTLGLIVPPHERKFEKKFIELVVVVDHSMVTKYNNDSTAIRTWIYEMLNTVNEIYL
           651   EPIKKTLGLIVPPHERKFEKKFIELVVVVDHSMVTKYNNDSTAIRTWIYEMLNTVNEIYL  830

AZ ecarin  241   PFNIRVALVGLEFWCNGDLINVTSTADDTLHSFGEWRASDLLNRKRHDHAQLLTNVTLDH  300
WT ecarin        PFNIRVALVGLEFWCNGDLINVTSTADDTLHSFGEWRASDLLNRKRHDHAQLLTNVTLDH
           831   PFNIRVALVGLEFWCNGDLINVTSTADDTLHSFGEWRASDLLNRKRHDHAQLLTNVTLDH  1010
```

AZ ecarin	301	STLGITFVYGMCKSDRSVELILDYSNITFNMAYIIAHEMGHSLGMLHDTKFCTCGAKPCI	360
WT ecarin		STLGITFVYGMCKSDRSVELILDYSNITFNMAYIIAHEMGHSLGMLHDTKFCTCGAKPCI	
	1011	STLGITFVYGMCKSDRSVELILDYSNITFNMAYIIAHEMGHSLGMLHDTKFCTCGAKPCI	1190
AZ ecarin	361	MFGKESIPPPKEFSSCSYDQYNKYLLKYNPKCILDPPLRKDIASPAVCGNEIWEEGEECD	420
WT ecarin		MFGKESIPPPKEFSSCSYDQYNKYLLKYNPKCILDPPLRKDIASPAVCGNEIWEEGEECD	
	1191	MFGKESIPPPKEFSSCSYDQYNKYLLKYNPKCILDPPLRKDIASPAVCGNEIWEEGEECD	1370
AZ ecarin	421	CGSPADCRNPCCDAATCKLKPGAECGNGECCDKCKIRKAGTECRPARDDCDVAEHCTGQS	480
WT ecarin		CGSPADCRNPCCDAATCKLKPGAECGNGECCDKCKIRKAGTECRPARDDCDVAEHCTGQS	
	1371	CGSPADCRNPCCDAATCKLKPGAECGNGECCDKCKIRKAGTECRPARDDCDVAEHCTGQS	1550
AZ ecarin	481	AECPRNEFQRNGQPCLNNSGYCYNGDCPIMLNQCIALFSPSATVAQDSCFQRNLQGSYYG	540
WT ecarin		AECPRNEFQRNGQPCLNNSGYCYNGDCPIMLNQCIALFSPSATVAQDSCFQRNLQGSYYG	
	1551	AECPRNEFQRNGQPCLNNSGYCYNGDCPIMLNQCIALFSPSATVAQDSCFQRNLQGSYYG	1730
AZ ecarin	541	YCTKEIGYYGKRFPCAPQDVKCGRLYCLDNSFKKNMRCKNDYSYADENKGIVEPGTKCED	600
WT ecarin		YCTKEIGYYGKRFPCAPQDVKCGRLYCLDNSFKKNMRCKNDYSYADENKGIVEPGTKCED	
	1731	YCTKEIGYYGKRFPCAPQDVKCGRLYCLDNSFKKNMRCKNDYSYADENKGIVEPGTKCED	1910
AZ ecarin	601	GKVCINRKCVDVNTAY	616
WT ecarin		GKVCINRKCVDVNTAY	
	1911	GKVCINRKCVDVNTAY	1958

Fig. 5

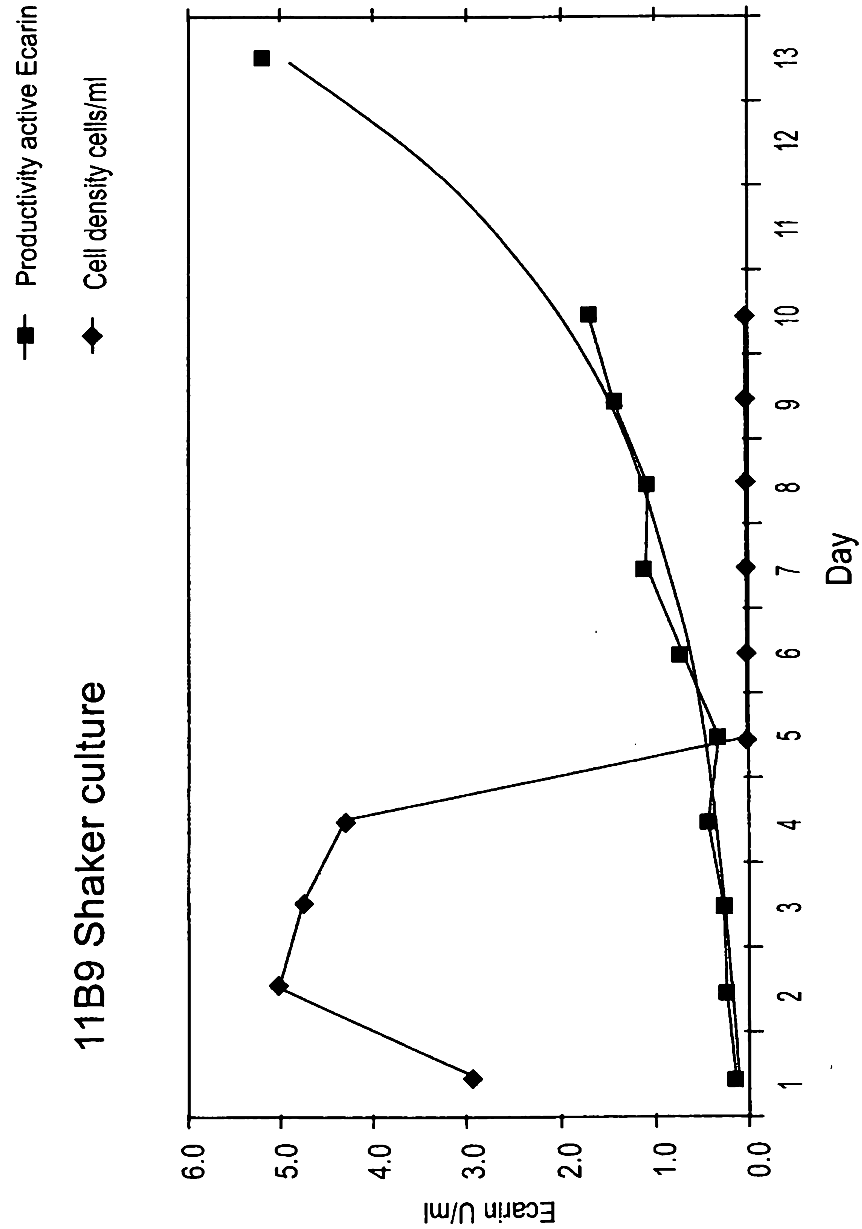

11B9 Shaker culture
Productivity active Ecarin U/ml
Cell density cells/ml
Ecarin U/ml
Cells/ml
Day
0.0
1.0
2.0
3.0
4.0
5.0
6.0
0
2000000
4000000
6000000
8000000
10000000
12000000
1
2
3
4
5
6
7
8
9
10
11
12
13

Fig. 6

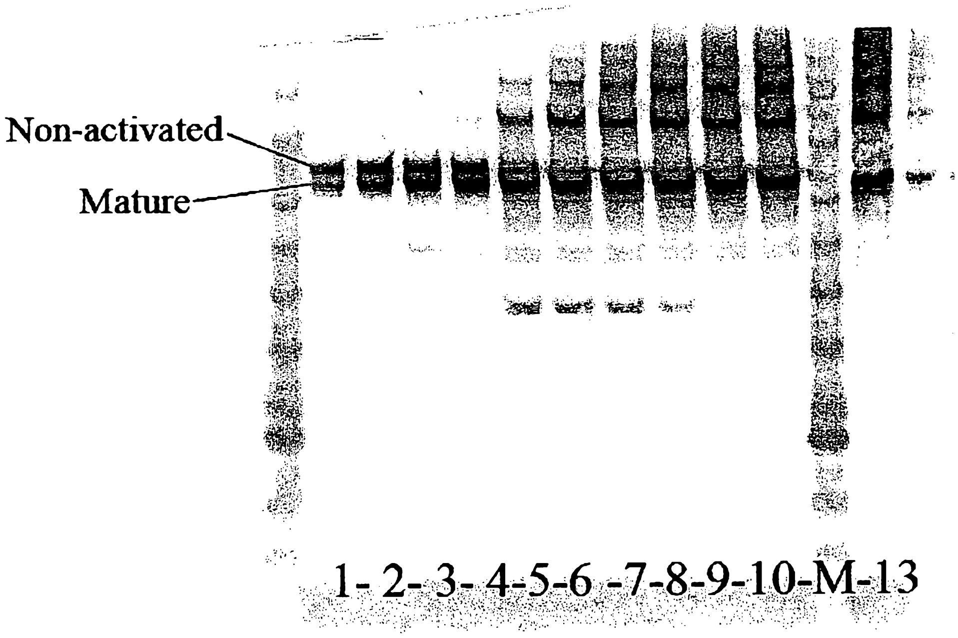
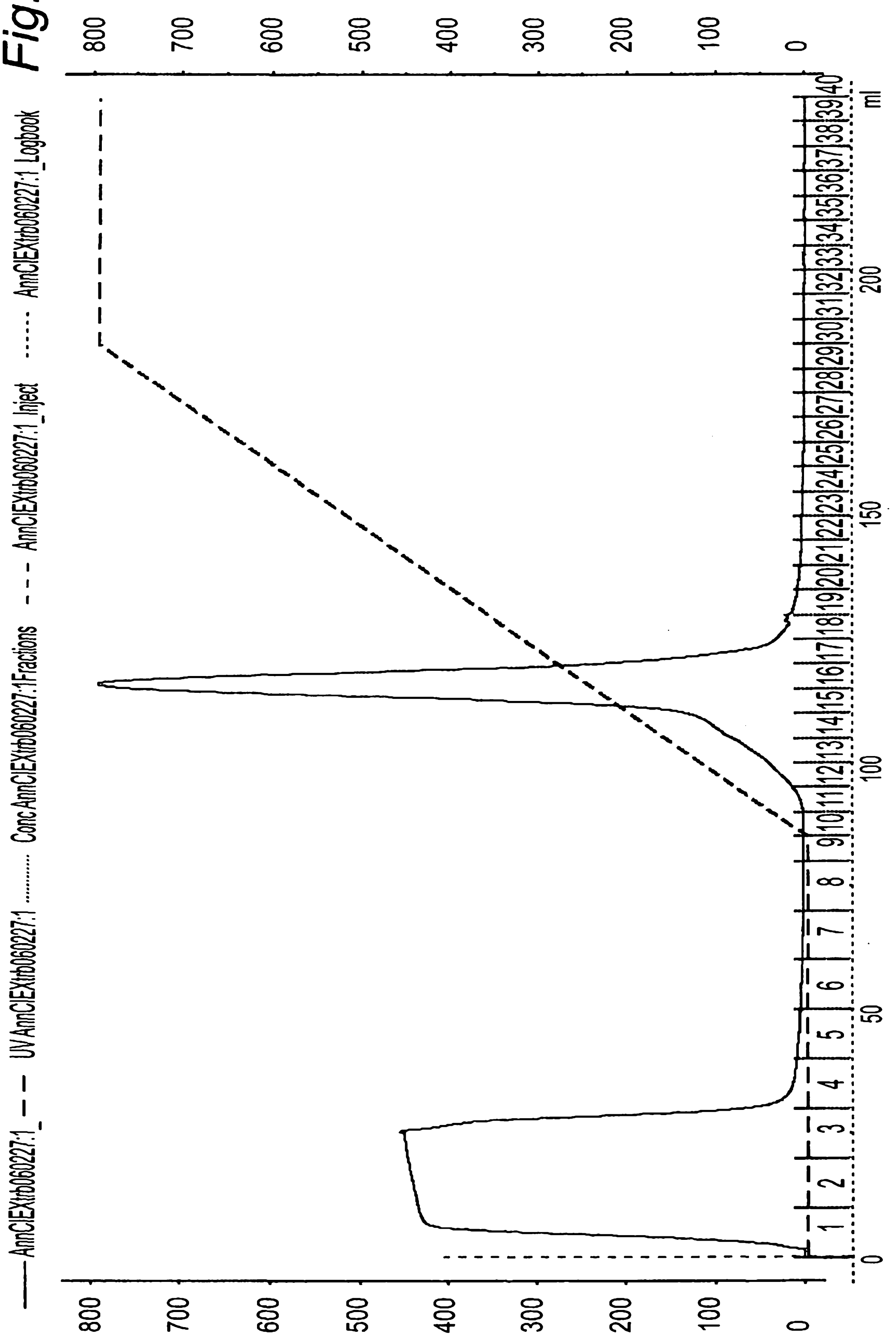


Fig. 7

Fig. 8


AnnCIEXtrb060227:1
UV AnnCIEXtrb060227:1
Conc AnnCIEXtrb060227:1Fractions
AnnCIEXtrb060227:1_Inject
AnnCIEXtrb060227:1_Logbook
ml

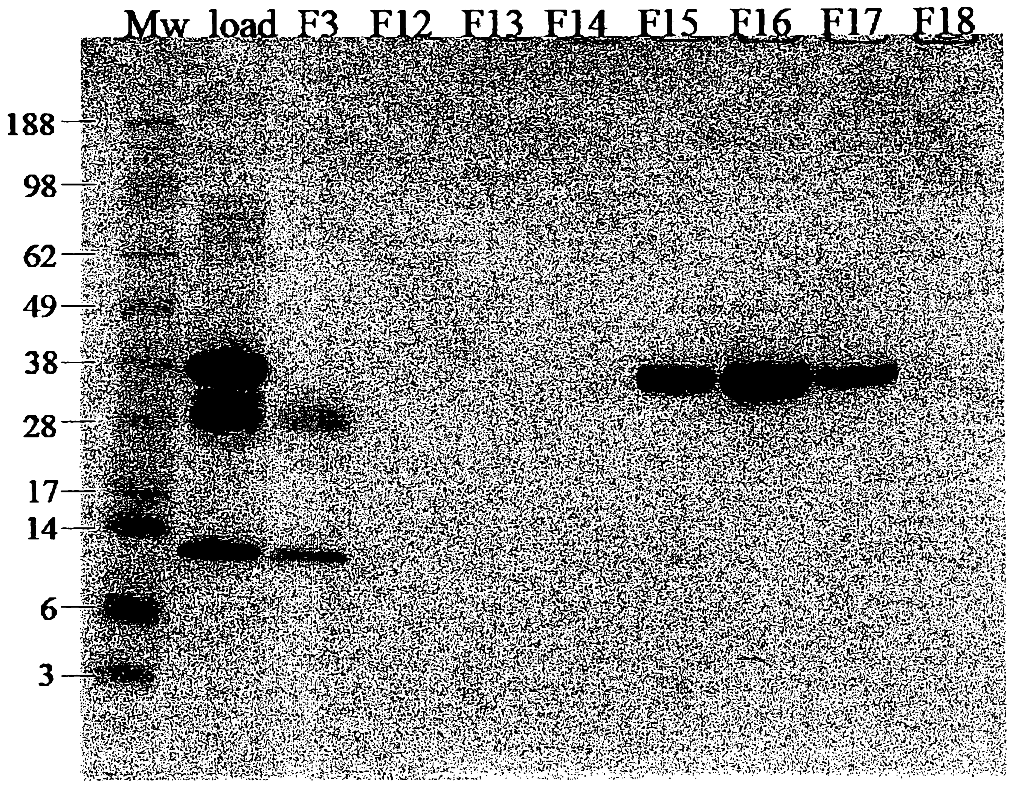


Fig. 9

SEQUENCE LISTING

<110> AstraZeneca AB

<120> METHOD FOR PRODUCTION OF RECOMBINANT HUMAN THROMBIN '644

<130> 30897304/EJH/RAS

<140> PCT/SE2008/050836
<141> 2008-07-04

<150> 60/948207
<151> 2007-07-06

<160> 5

<170> PatentIn version 3.5

<210> 1
<211> 10139
<212> DNA
<213> Synthetic

<400> 1
gacggatcgg gagatctccc gatcccctat ggtgcactct cagtacaatc tgctctgatg 60

ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120

cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180

ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240

gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300

tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360

cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420

attgacgtca atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt 480

atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540

atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600

tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660

actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720

aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780

gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840

ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc 900

gtttaaactt aagcttggta ccgagctcgg atccactagt ccagtgtggt ggaattgccc 960

ttattcctca gtgacccagg agctgacaca ctatggcgca cgtccgaggc ttgcagctgc 1020

ctggctgcct ggccctggct gccctgtgta gccttgtgca cagccagcat gtgttcctgg 1080
ctcctcagca agcacggtcg ctgctccagc gggtccggcg agccaacacc ttcttggagg 1140
aggtgcgcaa gggcaacctg gagcgagagt gcgtggagga gacgtgcagc tacgaggagg 1200
ccttcgaggc tctggagtcc tccacggcta cggatgtgtt ctgggccaag tacacagctt 1260
gtgagacagc gaggacgcct cgagataagc ttgctgcatg tctggaaggt aactgtgctg 1320
agggtctggg tacgaactac cgagggcatg tgaacatcac ccggtcaggc attgagtgcc 1380
agctatggag gagtcgctac ccacataagc ctgaaatcaa ctccactacc catcctgggg 1440
ccgacctaca ggagaatttc tgccgcaacc ccgacagcag caccacggga ccctggtgct 1500
acactacaga ccccaccgtg aggaggcagg aatgcagcat ccctgtctgt ggccaggatc 1560
aagtcactgt agcgatgact ccacgctccg aaggctccag tgtgaatctg tcacctccat 1620
tggagcagtg tgtccctgat cgggggcagc agtaccaggg gcgcctggcg gtgaccacac 1680
atgggctccc ctgcctggcc tgggccagcg cacaggccaa ggccctgagc aagcaccagg 1740
acttcaactc agctgtgcag ctggtggaga acttctgccg caacccagac ggggatgagg 1800
agggcgtgtg gtgctatgtg gccgggaagc ctggcgactt tgggtactgc gacctcaact 1860
attgtgagga ggccgtggag gaggagacag gagatgggct ggatgaggac tcagacaggg 1920
ccatcgaagg gcgtaccgcc accagtgagt accagacttt cttcaatccg aggacctttg 1980
gctcgggaga ggcagactgt gggctgcgac ctctgttcga gaagaagtcg ctggaggaca 2040
aaaccgaaag agagctcctg gaatcctaca tcgacgggcg cattgtggag ggctcggatg 2100
cagagatcgg catgtcacct tggcaggtga tgcttttccg gaagagtccc caggagctgc 2160
tgtgtggggc cagcctcatc agtgaccgct gggtcctcac cgccgcccac tgcctcctgt 2220
acccgccctg ggacaagaac ttcaccgaga atgaccttct ggtgcgcatt ggcaagcact 2280
cccgcaccag gtacgagcga aacattgaaa agatatccat gttggaaaag atctacatcc 2340
accccaggta caactggcgg gagaacctgg accgggacat tgccctgatg aagctgaaga 2400
agcctgttgc cttcagtgac tacattcacc ctgtgtgtct gcccgacagg gagacggcag 2460
ccagcttgct ccaggctgga tacaaggggc gggtgacagg ctggggcaac ctgaaggaga 2520
cgtggacagc caacgttggt aaggggcagc ccagtgtcct gcaggtggtg aacctgccca 2580
ttgtggagcg gccggtctgc aaggactcca cccggatccg catcactgac aacatgttct 2640
gtgctggtta caagcctgat gaagggaaac gaggggatgc ctgtgaaggt gacagtgggg 2700
gaccctttgt catgaagagc ccctttaaca accgctggta tcaaatgggc atcgtctcat 2760

ggggtgaagg ctgtgaccgg gatgggaaat atggcttcta cacacatgtg ttccgcctga 2820
agaagtggat acagaaggtc attgatcagt ttggagagta gaagggcaat tctgcagata 2880
tccagcacag tggcggccgc tcgagtctag agggcccgtt taaacccgct gatcagcctc 2940
gactgtgcct tctagttgcc agccatctgt tgtttgcccc tcccccgtgc cttccttgac 3000
cctggaaggt gccactccca ctgtcctttc ctaataaaat gaggaaattg catcgcattg 3060
tctgagtagg tgtcattcta ttctgggggg tggggtgggg caggacagca agggggagga 3120
ttgggaagac aatagcaggc atgctgggga tgcggtgggc tctatggctt ctgaggcgga 3180
aagaaccagc tggggctcta gggggtatcc ccacgcgccc tgtagcggcg cattaagcgc 3240
ggcgggtgtg gtggttacgc gcagcgtgac cgctacactt gccagcgccc tagcgcccgc 3300
tcctttcgct ttcttccctt cctttctcgc cacgttcgcc ggctttcccc gtcaagctct 3360
aaatcggggg ctccctttag ggttccgatt tagtgcttta cggcaccttc gaccccaaaa 3420
aacttgatta gggctgtgga atgtgtgtca gttagggtgt ggaaagtccc caggctcccc 3480
agcaggcaga agtatgcaaa gcatgcatct caattagtca gcaaccaggt gtggaaagtc 3540
cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt cagcaaccat 3600
agtcccgccc ctaactccgc ccatcccgcc cctaactccg cccagttccg cccattctcc 3660
gccccatggc tgactaattt tttttattta tgcagaggcc gaggccgcct cggcctctga 3720
gctattccag aagtagtgag gaggcttttt tggaggccta ggcttttgca aaaagctctc 3780
tggctaacta gagaacccac tgcttactgg cttatcgaaa ttaatacgac tcactatagg 3840
gagacccaag ctggctagcg tttaaactta agcttggtac cgagctcgga tccactagtc 3900
cagtgtggtg gaattgccct ttccgcagag caatggcggt gtctgccggg tccgcgcgga 3960
cctcgcccag ctcagataaa gtacagaaag acaaggctga actgatctca gggcccaggc 4020
aggacagccg aatagggaaa ctcttgggtt ttgagtggac agatttgtcc agttggcgga 4080
ggctggtgac cctgctgaat cgaccaacgg accctgcaag cttagctgtc tttcgttttc 4140
tttttgggtt cttgatggtg ctagacattc cccaggagcg gggctcagc tctctggacc 4200
ggaaatacct tgatgggctg gatgtgtgcc gcttcccctt gctggatgcc ctacgcccac 4260
tgccacttga ctggatgtat cttgtctaca ccatcatgtt tctgggggca ctgggcatga 4320
tgctgggcct gtgctaccgg ataagctgtg tgttattcct gctgccatac tggtatgtgt 4380
ttctcctgga caagacatca tggaacaacc actcctatct gtatgggttg ttggcctttc 4440
agctaacatt catggatgca aaccactact ggtctgtgga cggtctgctg aatgcccata 4500

2008275828 24 Aug 2012

ggaggaatgc ccacgtgccc ctttggaact atgcagtgct ccgtggccag atcttcattg 4560
tgtacttcat tgcgggtgtg aaaaagctgg atgcagactg ggttgaaggc tattccatgg 4620
aatatttgtc ccggcactgg ctcttcagtc ccttcaaact gctgttgtct gaggagctga 4680
ctagcctgct ggtcgtgcac tggggtgggc tgctgcttga cctctcagct ggtttcctgc 4740
tcttttttga tgtctcaaga tccattggcc tgttctttgt gtcctacttc cactgcatga 4800
attcccagct tttcagcatt ggtatgttct cctacgtcat gctggccagc agccctctct 4860
tctgctcccc tgagtggcct cggaagctgg tgtcctactg cccccgaagg ttgcaacaac 4920
tgttgcccct caaggcagcc cctcagccca gtgtttcctg tgtgtataag aggagccggg 4980
gcaaaagtgg ccagaagcca gggctgcgcc atcagctggg agctgccttc accctgctct 5040
acctcctgga gcagctattc ctgccctatt ctcattttct cacccagggc tataacaact 5100
ggacaaatgg gctgtatggc tattcctggg acatgatggt gcactcccgc tcccaccagc 5160
acgtgaagat cacctaccgt gatggccgca ctggcgaact gggctacctt aaccctgggg 5220
tatttacaca gagtcggcga tggaaggatc atgcagacat gctgaagcaa tatgccactt 5280
gcctgagccg cctgcttccc aagtataatg tcactgagcc ccagatctac tttgatattt 5340
gggtctccat caatgaccgc ttccagcaga ggatttttga ccctcgtgtg gacatcgtgc 5400
aggccgcttg gtcacccttt cagcgcacat cctgggtgca accactcttg atggacctgt 5460
ctccctggag ggccaagtta caggaaatca agagcagcct agacaaccac actgaggtgg 5520
tcttcattgc agatttccct ggactgcact tggagaattt tgtgagtgaa gacctgggca 5580
acactagcat ccagctgctg caggggggaag tgactgtgga gcttgtggca gaacagaaga 5640
accagactct tcgagaggga gaaaaaatgc agttgcctgc tggtgagtac cataaggtgt 5700
atacgacatc acctagccct tcttgctaca tgtacgtcta tgtcaacact acagagcttg 5760
cactggagca agacctggca tatctgcaag aattaaagga aaaggtggag aatggaagtg 5820
aaacagggcc tctaccccca gagctgcagc ctctgttgga aggggaagta aaagggggcc 5880
ctgagccaac acctctggtt cagacctttc ttagacgcca acaaaggctc caggagattg 5940
aacgccggcg aaatactcct ttccatgagc gattcttccg cttcttgttg cgaaagctct 6000
atgtctttcg ccgcagcttc ctgatgactt gtatctcact tcgaaatctg atattaggcc 6060
gtccttccct ggagcagctg gcccaggagg tgacttatgc aaacttgaga ccctttgagg 6120
cagttggaga actgaatccc tcaaacacgg attcttcaca ttctaatcct cctgagtcaa 6180
atcctgatcc tgtccactca gagttctgaa gggggccaga tgttggaagg gcaattcgag 6240

tctagagggc ccgccctgat agacggtttt tcgccctttg acgttggagt ccacgttctt 6300

taatagtgga ctcttgttcc aaactggaac aacactcaac cctatctcgg tctattcttt 6360

tgatttataa gggattttgc cgatttcggc ctattggtta aaaaatgagc tgatttaaca 6420

aaaatttaac gcgaattaat tctgtggaat gtgtgtcagt tagggtgtgg aaagtcccca 6480

ggctccccag caggcagaag tatgcaaagc atgcatctca attagtcagc aaccaggtgt 6540

ggaaagtccc caggctcccc agcaggcaga agtatgcaaa gcatgcatct caattagtca 6600

gcaaccatag tcccgcccct aactccgccc atcccgcccc taactccgcc cagttccgcc 6660

cattctccgc cccatggctg actaattttt tttatttatg cagaggccga ggccgcctct 6720

gcctctgagc tattccagaa gtagtgagga ggcttttttg gaggcctagg cttttgcaaa 6780

aagctcccgg gagcttgtat atccattttc ggatctgatc aagagacagg atgaggatcg 6840

tttcgcatga ttgaacaaga tggattgcac gcaggttctc cggccgcttg ggtggagagg 6900

ctattcggct atgactgggc acaacagaca atcggctgct ctgatgccgc cgtgttccgg 6960

ctgtcagcgc aggggcgccc ggttcttttt gtcaagaccg acctgtccgg tgccctgaat 7020

gaactgcagg acgaggcagc gcggctatcg tggctggcca cgacgggcgt tccttgcgca 7080

gctgtgctcg acgttgtcac tgaagcggga agggactggc tgctattggg cgaagtgccg 7140

gggcaggatc tcctgtcatc tcaccttgct cctgccgaga aagtatccat catggctgat 7200

gcaatgcggc ggctgcatac gcttgatccg gctacctgcc cattcgacca ccaagcgaaa 7260

catcgcatcg agcgagcacg tactcggatg gaagccggtc ttgtcgatca ggatgatctg 7320

gacgaagagc atcaggggct cgcgccagcc gaactgttcg ccaggctcaa ggcgcgcatg 7380

cccgacggcg aggatctcgt cgtgacccat ggcgatgcct gcttgccgaa tatcatggtg 7440

gaaaatggcc gcttttctgg attcatcgac tgtggccggc tgggtgtggc ggaccgctat 7500

caggacatag cgttggctac ccgtgatatt gctgaagagc ttggcggcga atgggctgac 7560

cgcttcctcg tgctttacgg tatcgccgct cccgattcgc agcgcatcgc cttctatcgc 7620

cttcttgacg agttcttctg agcgggactc tggggttcga aatgaccgac caagcgacgc 7680

ccaacctgcc atcacgagat ttcgattcca ccgccgcctt ctatgaaagg ttgggcttcg 7740

gaatcgtttt ccgggacgcc ggctggatga tcctccagcg cggggatctc atgctggagt 7800

tcttcgccca ccccaacttg tttattgcag cttataatgg ttacaaataa agcaatagca 7860

tcacaaattt cacaaataaa gcattttttt cactgcattc tagttgtggt ttgtccaaac 7920

tcatcaatgt atcttatcat gtctgtatac cgtcgacctc tagctagagc ttggcgtaat 7980

2008275828 24 Aug 2012


```
catggtcata gctgtttcct gtgtgaaatt gttatccgct cacaattcca cacaacatac    8040
gagccggaag cataaagtgt aaagcctggg gtgcctaatg agtgagctaa ctcacattaa    8100
ttgcgttgcg ctcactgccc gctttccagt cgggaaacct gtcgtgccag ctgcattaat    8160
gaatcggcca acgcgcgggg agaggcggtt tgcgtattgg gcgctcttcc gcttcctcgc    8220
tcactgactc gctgcgctcg gtcgttcggc tgcggcgagc ggtatcagct cactcaaagg    8280
cggtaatacg gttatccaca gaatcagggg ataacgcagg aaagaacatg tgagcaaaag    8340
gccagcaaaa ggccaggaac cgtaaaaagg ccgcgttgct ggcgtttttc cataggctcc    8400
gcccccctga cgagcatcac aaaaatcgac gctcaagtca gaggtggcga aacccgacag    8460
gactataaag ataccaggcg tttccccctg gaagctccct cgtgcgctct cctgttccga    8520
ccctgccgct taccggatac ctgtccgcct ttctcccttc gggaagcgtg gcgctttctc    8580
atagctcacg ctgtaggtat ctcagttcgg tgtaggtcgt tcgctccaag ctgggctgtg    8640
tgcacgaacc ccccgttcag cccgaccgct gcgccttatc cggtaactat cgtcttgagt    8700
ccaacccggt aagacacgac ttatcgccac tggcagcagc cactggtaac aggattagca    8760
gagcgaggta tgtaggcggt gctacagagt tcttgaagtg gtggcctaac tacggctaca    8820
ctagaagaac agtatttggt atctgcgctc tgctgaagcc agttaccttc ggaaaaagag    8880
ttggtagctc ttgatccggc aaacaaacca ccgctggtag cggttttttt gtttgcaagc    8940
agcagattac gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt tctacggggt    9000
ctgacgctca gtggaacgaa aactcacgtt aagggatttt ggtcatgaga ttatcaaaaa    9060
ggatcttcac ctagatcctt ttaaattaaa aatgaagttt taaatcaatc taaagtatat    9120
atgagtaaac ttggtctgac agttaccaat gcttaatcag tgaggcacct atctcagcga    9180
tctgtctatt tcgttcatcc atagttgcct gactccccgt cgtgtagata actacgatac    9240
gggagggctt accatctggc cccagtgctg caatgatacc gcgagaccca cgctcaccgg    9300
ctccagattt atcagcaata aaccagccag ccggaagggc cgagcgcaga agtggtcctg    9360
caactttatc cgcctccatc cagtctatta attgttgccg ggaagctaga gtaagtagtt    9420
cgccagttaa tagtttgcgc aacgttgttg ccattgctac aggcatcgtg gtgtcacgct    9480
cgtcgtttgg tatggcttca ttcagctccg gttcccaacg atcaaggcga gttacatgat    9540
cccccatgtt gtgcaaaaaa gcggttagct ccttcggtcc tccgatcgtt gtcagaagta    9600
agttggccgc agtgttatca ctcatggtta tggcagcact gcataattct cttactgtca    9660
tgccatccgt aagatgcttt tctgtgactg gtgagtactc aaccaagtca ttctgagaat    9720
```

agtgtatgcg gcgaccgagt tgctcttgcc cggcgtcaat acgggataat accgcgccac 9780

atagcagaac tttaaaagtg ctcatcattg gaaaacgttc ttcggggcga aaactctcaa 9840

ggatcttacc gctgttgaga tccagttcga tgtaacccac tcgtgcaccc aactgatctt 9900

cagcatcttt tactttcacc agcgtttctg ggtgagcaaa aacaggaagg caaaatgccg 9960

caaaaaaggg aataagggcg acacggaaat gttgaatact catactcttc ctttttcaat 10020

attattgaag catttatcag ggttattgtc tcatgagcgg atacatattt gaatgtattt 10080

agaaaaataa acaaataggg gttccgcgca catttccccg aaaagtgcca cctgacgtc 10139

<210> 2
<211> 1851
<212> DNA
<213> Synthetic

<400> 2
atgatccaga tcctgctggt gatcatctgc ctggccgtgt tcccctacca gggctgctcc 60

atcatcctgg gcagcggcaa cgtgaacgac tacgaggtgg tgtaccccca gaaggtgacc 120

gccctgccca agggcgccgt gcagcagccc gagcagaaat acgaggacgc catgcagtac 180

gagttcgagg tgaagggcga gcccgtggtg ctgcacctgg agaagaacaa ggagctgttc 240

agcgaggact acagcgagac ccactacagc agcgacgaca gggagatcac caccaacccc 300

agcgtggagg accactgcta ctaccacggc cggatccaga acgacgccga gagcaccgcc 360

agcatcagcg cctgtaatgg cctgaagggc cacttcaagc tgagaggcga gacctacttc 420

atcgagcccc tgaagatccc cgacagcgag gcccacgccg tgtacaagta cgagaacatc 480

gagaacgagg acgaggcccc taagatgtgt ggcgtgaccc aggacaactg ggagagcgac 540

gagcccatca agaaaaccct gggcctgatc gtgccccccc acgagagaaa gttcgagaag 600

aagttcatcg aactggtggt cgtggtggac cacagcatgg tgaccaagta caacaacgac 660

agcaccgcca tcaggacctg gatctacgag atgctgaaca ccgtgaacga gatctacctg 720

cccttcaaca tcagagtggc cctggtgggc ctggagttct ggtgtaacgg cgacctgatc 780

aacgtgacca gcaccgccga cgacaccctg cacagcttcg gcgagtggag agccagcgac 840

ctgctgaacc ggaagagaca cgatcacgcc cagctgctga ccaatgtgac cctggaccac 900

tccaccctgg gcatcacctt cgtgtacggc atgtgtaaga gcgaccggag cgtggagctg 960

atcctggact acagcaacat caccttcaac atggcctaca tcatcgccca cgagatgggc 1020

cacagcctgg gcatgctgca cgacaccaag ttctgtacct gtggcgccaa gccctgtatc 1080

atgttcggca aggagagcat ccctcccccct aaggagttca gcagctgctc ctacgaccag 1140

2008275828 24 Aug 2012

tacaataagt acctgctgaa gtacaacccc aagtgtatcc tggaccccc cctgagaaag 1200

gacatcgcca gccctgccgt gtgtggcaat gagatctggg aggagggcga ggagtgtgac 1260

tgtggcagcc cagccgactg tagaaacccc tgctgtgatg ccgccacctg taagctgaag 1320

cctggcgccg agtgtggcaa cggcgagtgc tgtgacaagt gtaagatccg gaaggccggc 1380

accgagtgta gacccgccag ggacgattgt gacgtggccg agcactgtac cggccagagc 1440

gccgagtgcc ccagaaacga gttccagagg aacggccagc cttgcctgaa caacagcggc 1500

tactgctaca acggcgactg ccccatcatg ctgaaccagt gtatcgccct gttcagcccc 1560

agcgccaccg tggcccagga cagctgcttc cagagaaacc tgcagggcag ctactacggc 1620

tactgtacca aggagatcgg ctactacgga aagaggttcc cctgtgcccc tcaggacgtg 1680

aagtgtggca ggctgtactg cctggacaac tccttcaaga aaaacatgag gtgtaagaac 1740

gactacagct acgccgacga gaacaagggc atcgtggagc ccggcaccaa gtgtgaggac 1800

ggcaaagtgt gtatcaaccg gaagtgtgtg gacgtgaaca ccgcctactg a 1851

<210> 3
<211> 7280
<212> DNA
<213> Synthetic

<400> 3
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60

ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120

cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180

ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240

gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300

tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360

cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420

attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480

atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540

atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600

tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660

actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720

aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780

24 Aug 2012 2008275828

gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840
ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc 900
gtttaaactt aagcttggta ccgagctcgg atccactagt ccagtgtggt ggaattctgc 960
agatatccac catgatccag atcctgctgg tgatcatctg cctggccgtg ttcccctacc 1020
agggctgctc catcatcctg ggcagcggca acgtgaacga ctacgaggtg gtgtaccccc 1080
agaaggtgac cgccctgccc aagggcgccg tgcagcagcc cgagcagaaa tacgaggacg 1140
ccatgcagta cgagttcgag gtgaagggcg agcccgtggt gctgcacctg gagaagaaca 1200
aggagctgtt cagcgaggac tacagcgaga cccactacag cagcgacgac agggagatca 1260
ccaccaaccc cagcgtggag gaccactgct actaccacgg ccggatccag aacgacgccg 1320
agagcaccgc cagcatcagc gcctgtaatg gcctgaaggg ccacttcaag ctgagaggcg 1380
agacctactt catcgagccc ctgaagatcc ccgacagcga ggcccacgcc gtgtacaagt 1440
acgagaacat cgagaacgag gacgaggccc ctaagatgtg tggcgtgacc caggacaact 1500
gggagagcga cgagcccatc aagaaaaccc tgggcctgat cgtgcccccc cacgagagaa 1560
agttcgagaa gaagttcatc gaactggtgg tcgtggtgga ccacagcatg gtgaccaagt 1620
acaacaacga cagcaccgcc atcaggacct ggatctacga gatgctgaac accgtgaacg 1680
agatctacct gcccttcaac atcagagtgg ccctggtggg cctggagttc tggtgtaacg 1740
gcgacctgat caacgtgacc agcaccgccg acgacaccct gcacagcttc ggcgagtgga 1800
gagccagcga cctgctgaac cggaagagac acgatcacgc ccagctgctg accaatgtga 1860
ccctggacca ctccaccctg ggcatcacct tcgtgtacgg catgtgtaag agcgaccgga 1920
gcgtggagct gatcctggac tacagcaaca tcaccttcaa catggcctac atcatcgccc 1980
acgagatggg ccacagcctg ggcatgctgc acgacaccaa gttctgtacc tgtggcgcca 2040
agccctgtat catgttcggc aaggagagca tccctccccc taaggagttc agcagctgct 2100
cctacgacca gtacaataag tacctgctga agtacaaccc caagtgtatc ctggaccccc 2160
ccctgagaaa ggacatcgcc agccctgccg tgtgtggcaa tgagatctgg gaggagggcg 2220
aggagtgtga ctgtggcagc ccagccgact gtagaaaccc ctgctgtgat gccgccacct 2280
gtaagctgaa gcctggcgcc gagtgtggca acggcgagtg ctgtgacaag tgtaagatcc 2340
ggaaggccgg caccgagtgt agacccgcca gggacgattg tgacgtggcc gagcactgta 2400
ccggccagag cgccgagtgc cccagaaacg agttccagag gaacggccag ccttgcctga 2460
acaacagcgg ctactgctac aacggcgact gccccatcat gctgaaccag tgtatcgccc 2520

2008275828 24 Aug 2012

tgttcagccc cagcgccacc gtggcccagg acagctgctt ccagagaaac ctgcagggca 2580

gctactacgg ctactgtacc aaggagatcg gctactacgg aaagaggttc ccctgtgccc 2640

ctcaggacgt gaagtgtggc aggctgtact gcctggacaa ctccttcaag aaaaacatga 2700

ggtgtaagaa cgactacagc tacgccgacg agaacaaggg catcgtggag cccggcacca 2760

agtgtgagga cggcaaagtg tgtatcaacc ggaagtgtgt ggacgtgaac accgcctact 2820

gatgagcggc cgctcgagtc tagagggccc gtttaaaccc gctgatcagc ctcgactgtg 2880

ccttctagtt gccagccatc tgttgtttgc ccctcccccg tgccttcctt gaccctggaa 2940

ggtgccactc ccactgtcct ttcctaataa aatgaggaaa ttgcatcgca ttgtctgagt 3000

aggtgtcatt ctattctggg gggtggggtg gggcaggaca gcaaggggga ggattgggaa 3060

gacaatagca ggcatgctgg ggatgcggtg ggctctatgg cttctgaggc ggaaagaacc 3120

agctggggct ctagggggta tccccacgcg ccctgtagcg gcgcattaag cgcggcgggt 3180

gtggtggtta cgcgcagcgt gaccgctaca cttgccagcg ccctagcgcc cgctcctttc 3240

gctttcttcc cttcctttct cgccacgttc gccggctttc cccgtcaagc tctaaatcgg 3300

ggcatccctt tagggttccg atttagtgct ttacggcacc tcgaccccaa aaaacttgat 3360

tagggtgatg gttcacgtag tgggccatcg ccctgataga cggtttttcg ccctttgacg 3420

ttggagtcca cgttctttaa tagtggactc ttgttccaaa ctggaacaac actcaaccct 3480

atctcggtct attcttttga tttataaggg attttgggga tttcggccta ttggttaaaa 3540

aatgagctga tttaacaaaa atttaacgcg aattaattct gtggaatgtg tgtcagttag 3600

ggtgtggaaa gtccccaggc tccccaggca ggcagaagta tgcaaagcat gcatctcaat 3660

tagtcagcaa ccaggtgtgg aaagtcccca ggctccccag caggcagaag tatgcaaagc 3720

atgcatctca attagtcagc aaccatagtc ccgcccctaa ctccgcccat cccgccccta 3780

actccgccca gttccgccca ttctccgccc catggctgac taattttttt tatttatgca 3840

gaggccgagg ccgcctctgc ctctgagcta ttccagaagt agtgaggagg cttttttgga 3900

ggcctaggct tttgcaaaaa gctcccggga gcttgtatat ccattttcgg atctgatcaa 3960

gagacaggat gaggatcgtt tcgcatgatt gaacaagatg gattgcacgc aggttctccg 4020

gccgcttggg tggagaggct attcggctat gactgggcac aacagacaat cggctgctct 4080

gatgccgccg tgttccggct gtcagcgcag gggcgcccgg ttctttttgt caagaccgac 4140

ctgtccggtg ccctgaatga actgcaggac gaggcagcgc ggctatcgtg gctggccacg 4200

acgggcgttc cttgcgcagc tgtgctcgac gttgtcactg aagcgggaag ggactggctg 4260

```
ctattgggcg aagtgccggg gcaggatctc ctgtcatctc accttgctcc tgccgagaaa    4320
gtatccatca tggctgatgc aatgcggcgg ctgcatacgc ttgatccggc tacctgccca    4380
ttcgaccacc aagcgaaaca tcgcatcgag cgagcacgta ctcggatgga agccggtctt    4440
gtcgatcagg atgatctgga cgaagagcat caggggctcg cgccagccga actgttcgcc    4500
aggctcaagg cgcgcatgcc cgacggcgag gatctcgtcg tgacccatgg cgatgcctgc    4560
ttgccgaata tcatggtgga aaatggccgc ttttctggat tcatcgactg tggccggctg    4620
ggtgtggcgg accgctatca ggacatagcg ttggctaccc gtgatattgc tgaagagctt    4680
ggcggcgaat gggctgaccg cttcctcgtg ctttacggta tcgccgctcc cgattcgcag    4740
cgcatcgcct tctatcgcct tcttgacgag ttcttctgag cgggactctg gggttcgaaa    4800
tgaccgacca agcgacgccc aacctgccat cacgagattt cgattccacc gccgccttct    4860
atgaaaggtt gggcttcgga atcgttttcc gggacgccgg ctggatgatc ctccagcgcg    4920
gggatctcat gctggagttc ttcgcccacc ccaacttgtt tattgcagct tataatggtt    4980
acaaataaag caatagcatc acaaatttca caaataaagc atttttttca ctgcattcta    5040
gttgtggttt gtccaaactc atcaatgtat cttatcatgt ctgtataccg tcgacctcta    5100
gctagagctt ggcgtaatca tggtcatagc tgtttcctgt gtgaaattgt tatccgctca    5160
caattccaca caacatacga gccggaagca taaagtgtaa agcctggggt gcctaatgag    5220
tgagctaact cacattaatt gcgttgcgct cactgcccgc tttccagtcg ggaaacctgt    5280
cgtgccagct gcattaatga atcggccaac gcgcggggag aggcggtttg cgtattgggc    5340
gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg cggcgagcgg    5400
tatcagctca ctcaaaggcg gtaatacggt tatccacaga atcaggggat aacgcaggaa    5460
agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg taaaaaggcc gcgttgctgg    5520
cgtttttcca taggctccgc ccccctgacg agcatcacaa aaatcgacgc tcaagtcaga    5580
ggtggcgaaa cccgacagga ctataaagat accaggcgtt tccccctgga agctccctcg    5640
tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt ctcccttcgg    5700
gaagcgtggc gctttctcaa tgctcacgct gtaggtatct cagttcggtg taggtcgttc    5760
gctccaagct gggctgtgtg cacgaacccc ccgttcagcc cgaccgctgc gccttatccg    5820
gtaactatcg tcttgagtcc aacccggtaa gacacgactt atcgccactg gcagcagcca    5880
ctggtaacag gattagcaga gcgaggtatg taggcggtgc tacagagttc ttgaagtggt    5940
ggcctaacta cggctacact agaaggacag tatttggtat ctgcgctctg ctgaagccag    6000
```

ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc gctggtagcg 6060

gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa aaaaggatct caagaagatc 6120

ctttgatctt ttctacgggg tctgacgctc agtggaacga aaactcacgt taagggattt 6180

tggtcatgag attatcaaaa aggatcttca cctagatcct tttaaattaa aaatgaagtt 6240

ttaaatcaat ctaaagtata tatgagtaaa cttggtctga cagttaccaa tgcttaatca 6300

gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc tgactccccg 6360

tcgtgtagat aactacgata cgggagggct taccatctgg ccccagtgct gcaatgatac 6420

cgcgagaccc acgctcaccg gctccagatt tatcagcaat aaaccagcca gccggaaggg 6480

ccgagcgcag aagtggtcct gcaactttat ccgcctccat ccagtctatt aattgttgcc 6540

gggaagctag agtaagtagt tcgccagtta atagtttgcg caacgttgtt gccattgcta 6600

caggcatcgt ggtgtcacgc tcgtcgtttg gtatggcttc attcagctcc ggttcccaac 6660

gatcaaggcg agttacatga tcccccatgt tgtgcaaaaa agcggttagc tccttcggtc 6720

ctccgatcgt tgtcagaagt aagttggccg cagtgttatc actcatggtt atggcagcac 6780

tgcataattc tcttactgtc atgccatccg taagatgctt ttctgtgact ggtgagtact 6840

caaccaagtc attctgagaa tagtgtatgc ggcgaccgag ttgctcttgc ccggcgtcaa 6900

tacgggataa taccgcgcca catagcagaa ctttaaaagt gctcatcatt ggaaaacgtt 6960

cttcggggcg aaaactctca aggatcttac cgctgttgag atccagttcg atgtaaccca 7020

ctcgtgcacc caactgatct tcagcatctt ttactttcac cagcgtttct gggtgagcaa 7080

aaacaggaag gcaaaatgcc gcaaaaaagg gaataagggc gacacggaaa tgttgaatac 7140

tcatactctt cctttttcaa tattattgaa gcatttatca gggttattgt ctcatgagcg 7200

gatacatatt tgaatgtatt tagaaaaata aacaaatagg ggttccgcgc acatttcccc 7260

gaaaagtgcc acctgacgtc 7280

<210> 4
<211> 1848
<212> DNA
<213> echis carinatus

<400> 4
atgatccaga ttctcttggt aattatatgc ttagcagttt ttccatatca aggttgctct 60

ataatcctgg gatctgggaa tgttaatgat tatgaagtag tgtatccaca aaaagtcact 120

gcattgccca aaggagcagt tcagcagcct gagcaaaagt atgaagatgc catgcaatat 180

gaatttgaag tgaagggaga gccagtggtc cttcacctag aaaaaaataa agaacttttt 240

```
tcagaagatt acagtgagac tcattattcg tctgatgaca gagaaattac aacaaaccct    300
tcagttgagg atcactgcta ttatcatgga cggatccaga atgatgctga gtcaactgca    360
agcatcagtg catgcaatgg tttgaaagga catttcaagc ttcgagggga gacgtacttt    420
attgaaccct tgaagattcc cgacagtgaa gcccatgcag tctacaaata tgaaaacata    480
gaaaatgagg atgaagcccc caaaatgtgt ggggtaaccc aggataattg ggaatcagat    540
gaacccatca aaaagacttt ggggttaatt gttcctcctc atgaacgaaa atttgagaaa    600
aaattcattg agcttgtcgt agttgtggac cacagtatgg tcacaaaata caacaatgat    660
tcaactgcta taagaacatg gatatatgaa atgctcaaca ctgtaaatga gatatactta    720
cctttcaata ttcgtgtagc actggttggc ctagaatttt ggtgcaatgg agacttgatt    780
aacgtgacat ccacagcaga tgatactttg cactcatttg gagaatggag agcatcagat    840
ttgctgaatc gaaaaagaca tgatcatgct cagttactca cgaacgtgac actggatcat    900
tccactcttg gaatcacgtt cgtatatggc atgtgcaaat cagatcgttc tgtagaactt    960
attctggatt acagcaacat aacttttaat atggcatata taatagccca tgagatgggt   1020
catagtctgg gcatgttaca tgacacaaaa ttctgtactt gtggggctaa accatgcatt   1080
atgtttggca aagaaagcat tccaccgccc aaagaattca gcagttgtag ttatgaccag   1140
tataacaagt atcttcttaa atataaccca aaatgcattc ttgattcacc tttgagaaaa   1200
gatattgctt cacctgcagt ttgtggaaat gaaatttggg aggaaggaga agaatgtgat   1260
tgtggttctc ctgcagattg tcgaaatcca tgctgtgatg ctgcaacatg taaactgaaa   1320
ccaggggcag aatgtggaaa tggagagtgt tgtgacaagt gcaagattag gaaagcagga   1380
acagaatgcc ggccagcaag ggatgactgt gatgtcgctg aacactgcac tggccaatct   1440
gctgagtgtc ccagaaatga gttccaaagg aatggacaac catgccttaa caactcgggt   1500
tattgctaca atggggattg ccccatcatg ttaaaccaat gtattgctct ctttagtcca   1560
agtgcaactg tggctcaaga ttcatgtttt cagaggaact tgcaaggcag ttactatggc   1620
tactgcacaa aggaaattgg ttactatggt aaaaggtttc catgtgcacc acaagatgta   1680
aaatgtggca gattatactg cttagataat tcattcaaaa aaaatatgcg ttgcaagaac   1740
gactattcat acgcggatga aaataaggga atagttgaac ctggaacaaa atgtgaagat   1800
ggaaaggtct gcatcaacag gaagtgtgtt gatgtgaata cagcctac                1848
```

<210> 5
<211> 616

<212> PRT
<213> Kenyan Echis carinatus

<400> 5

```
Met Ile Gln Ile Leu Leu Val Ile Ile Cys Leu Ala Val Phe Pro Tyr
1               5                   10                  15

Gln Gly Cys Ser Ile Ile Leu Gly Ser Gly Asn Val Asn Asp Tyr Glu
            20                  25                  30

Val Val Tyr Pro Gln Lys Val Thr Ala Leu Pro Lys Gly Ala Val Gln
        35                  40                  45

Gln Pro Glu Gln Lys Tyr Glu Asp Ala Met Gln Tyr Glu Phe Glu Val
    50                  55                  60

Lys Gly Glu Pro Val Val Leu His Leu Glu Lys Asn Lys Glu Leu Phe
65                  70                  75                  80

Ser Glu Asp Tyr Ser Glu Thr His Tyr Ser Ser Asp Asp Arg Glu Ile
                85                  90                  95

Thr Thr Asn Pro Ser Val Glu Asp His Cys Tyr Tyr His Gly Arg Ile
            100                 105                 110

Gln Asn Asp Ala Glu Ser Thr Ala Ser Ile Ser Ala Cys Asn Gly Leu
        115                 120                 125

Lys Gly His Phe Lys Leu Arg Gly Glu Thr Tyr Phe Ile Glu Pro Leu
    130                 135                 140

Lys Ile Pro Asp Ser Glu Ala His Ala Val Tyr Lys Tyr Glu Asn Ile
145                 150                 155                 160

Glu Asn Glu Asp Glu Ala Pro Lys Met Cys Gly Val Thr Gln Asp Asn
                165                 170                 175

Trp Glu Ser Asp Glu Pro Ile Lys Lys Thr Leu Gly Leu Ile Val Pro
            180                 185                 190

Pro His Glu Arg Lys Phe Glu Lys Lys Phe Ile Glu Leu Val Val Val
        195                 200                 205

Val Asp His Ser Met Val Thr Lys Tyr Asn Asn Asp Ser Thr Ala Ile
    210                 215                 220

Arg Thr Trp Ile Tyr Glu Met Leu Asn Thr Val Asn Glu Ile Tyr Leu
225                 230                 235                 240

Pro Phe Asn Ile Arg Val Ala Leu Val Gly Leu Glu Phe Trp Cys Asn
                245                 250                 255

Gly Asp Leu Ile Asn Val Thr Ser Thr Ala Asp Asp Thr Leu His Ser
            260                 265                 270

Phe Gly Glu Trp Arg Ala Ser Asp Leu Leu Asn Arg Lys Arg His Asp
        275                 280                 285
```

His Ala Gln Leu Leu Thr Asn Val Thr Leu Asp His Ser Thr Leu Gly
290 295 300

Ile Thr Phe Val Tyr Gly Met Cys Lys Ser Asp Arg Ser Val Glu Leu
305 310 315 320

Ile Leu Asp Tyr Ser Asn Ile Thr Phe Asn Met Ala Tyr Ile Ile Ala
325 330 335

His Glu Met Gly His Ser Leu Gly Met Leu His Asp Thr Lys Phe Cys
340 345 350

Thr Cys Gly Ala Lys Pro Cys Ile Met Phe Gly Lys Glu Ser Ile Pro
355 360 365

Pro Pro Lys Glu Phe Ser Ser Cys Ser Tyr Asp Gln Tyr Asn Lys Tyr
370 375 380

Leu Leu Lys Tyr Asn Pro Lys Cys Ile Leu Asp Pro Pro Leu Arg Lys
385 390 395 400

Asp Ile Ala Ser Pro Ala Val Cys Gly Asn Glu Ile Trp Glu Glu Gly
405 410 415

Glu Glu Cys Asp Cys Gly Ser Pro Ala Asp Cys Arg Asn Pro Cys Cys
420 425 430

Asp Ala Ala Thr Cys Lys Leu Lys Pro Gly Ala Glu Cys Gly Asn Gly
435 440 445

Glu Cys Cys Asp Lys Cys Lys Ile Arg Lys Ala Gly Thr Glu Cys Arg
450 455 460

Pro Ala Arg Asp Asp Cys Asp Val Ala Glu His Cys Thr Gly Gln Ser
465 470 475 480

Ala Glu Cys Pro Arg Asn Glu Phe Gln Arg Asn Gly Gln Pro Cys Leu
485 490 495

Asn Asn Ser Gly Tyr Cys Tyr Asn Gly Asp Cys Pro Ile Met Leu Asn
500 505 510

Gln Cys Ile Ala Leu Phe Ser Pro Ser Ala Thr Val Ala Gln Asp Ser
515 520 525

Cys Phe Gln Arg Asn Leu Gln Gly Ser Tyr Tyr Gly Tyr Cys Thr Lys
530 535 540

Glu Ile Gly Tyr Tyr Gly Lys Arg Phe Pro Cys Ala Pro Gln Asp Val
545 550 555 560

Lys Cys Gly Arg Leu Tyr Cys Leu Asp Asn Ser Phe Lys Lys Asn Met
565 570 575

Arg Cys Lys Asn Asp Tyr Ser Tyr Ala Asp Glu Asn Lys Gly Ile Val
580 585 590

```
Glu Pro Gly Thr Lys Cys Glu Asp Gly Lys Val Cys Ile Asn Arg Lys
        595                 600                 605

Cys Val Asp Val Asn Thr Ala Tyr
    610                 615
```