This invention provides a new class of enhanced apyrases (EN-apyrases) with superior pharmacokinetic, pharmacodynamic, and pharmacocchemical properties and which can be purified using simplified procedures. The invention further provides constructs for transforming a cell to produce these EN-apyrases. The EN-apyrase construct comprises sequences encoding a signal sequence, a linker, and a soluble apyrase. Also provided are preparations of apyrases and methods for producing apyrase in culture cells and purification thereof.
THERAPEUTIC APYRASE CONSTRUCTS, APYRASE AGENTS, AND PRODUCTION METHODS

Cross-Reference to Related Application

[0001] This application claims benefit of U.S. Serial No. 61/294,695 filed 13 January 2010. The contents of this document are incorporated herein by reference.

Technical Field

[0002] The present invention relates to novel apyrases and uses thereof to treat thrombotic or inflammation-related diseases.

Background

[0003] Apyrases (Ecto-ATP diphosphohydrolases) constitute a group of enzymes catalyzing metabolism of ATP to ADP and ADP to AMP. The first known human apyrase, CD39, was originally identified as a cell-surface protein on activated lymphocytes and endothelial cells. Both the in vitro and in vivo studies clearly indicated that CD39 represents an important apyrase in cardiovascular health by regulating levels of ADP. For example, apyrase is known to inhibit platelet aggregation by metabolizing extracellular ADP. Different from clopidogrel (Plavix) strategies that irreversibly bind to ADP receptor on the platelet, human apyrase does not damage the platelets per se or interfere with normal platelet function providing a safer approach to patients with excessive platelet activation.


[0005] Specifically human CD39L3 is a 529 amino acid protein shown in SEQ ID NO:1 with a predicted molecular weight of 59132.42 Daltons. The isoelectric point of CD39L3 is 6.233. There are seven putative glycosylation sites and 13 cysteine residues. Based on SEQ ID NO:1, the N-terminal 43 residues and C-terminal 44 residues are considered to be part of a transmembrane domain. The catalytic core of the enzyme roughly resides between amino acid 44 through amino acid 238, and soluble forms of this protein and related apyrases containing these residues have been prepared and described by Chen, et al. (U.S. Pat No. 7,247,300). Additionally, substituting an arginine for a glycine at residue 67 and/or a threonine for an...
arginine at residue 69 is shown to confer additional desired properties including enhanced ADP'ase activity where the residue number refers to the wild-type human CD39L3 shown as SEQ ID NO:1, as described in U.S. 7,390,485.

[0006] ProtParam analysis shows that both CD39L3 and CD39 are composed of about 520 amino acids with the pi of about 6.0. CD39L3 and CD39 also share similar amino acid compositions to each other and common structural motifs including about 440 amino acid residues of the extracellular ATP/ADPase portion that resides between the N- and C-terminal transmembrane regions. Although CD39L3 is found in chromosome 3 and CD39 in chromosome 10, their overall intron and exon structures are identical with 10 exons each.

[0007] Bioinformatics analysis suggests that CD39L3 is a brain specific isozyme or isoenzyme of CD39. Isoenzymes or isoenzymes may not have the same regulatory properties of their respective counterpart, but rather have adjusted their enzymatic properties to be optimal for the precise environment to which they are subjected. Northern blot studies showed CD39L3 is highly expressed in brain and kidney, while CD39 is expressed in placenta and spleen. The analysis suggests that expression of the isoenzyme CD39L3 in human brain complements the activity of CD39 as the key thromboregulator.

[0008] The present invention provides a new class of apyrases compounds and preparations thereof with improved therapeutic properties such as longer half-life, higher stability, or higher solubility, or higher purity. Methods of making such improved preparations at a high concentration in a form that can be readily purified to substantial homogeneity are also disclosed.

Disclosure of the Invention

[0009] A new class of apyrases ("EN-apyrase", for "enhanced apyrase") has been prepared that has superior pharmacokinetic properties and that is more easily purified from culture.

[0010] It has been found that appropriate design of an expression vector wherein a nucleotide sequence encoding a soluble form of CD39L3 glycoprotein is appropriately coupled to a signal sequence and expressed in Chinese hamster ovary cells under suitable conditions, enhanced forms of a apyrase are obtained. These enhanced apyrase are characterized by a lower isoelectric point presumably due to enhanced glycosylation and sialation and also by uniform cleavage at the N-terminus thus easing purification and providing a more homogenous sample. Typically, the EN-apyrase is a soluble form of SEQ ID NO:1, including mutants at position 67 and/or 69. The soluble forms span approximately positions 49 through position 485 of SEQ ID
NO:1 and the foregoing mutants. Typically, they have isoelectric points in the range of 3-4.5 and are heavily glycosylated.

[0011] Thus in one aspect, the invention is directed to an EN-apyrase, wherein the EN-apyrase is a soluble CD39L3 or a homolog thereof, has a homogeneous N-terminus and has an average isoelectric point in the range of about 3.0 to about 4.5; and/or wherein said EN-apyrase has an in vivo half-life in rabbits or pigs at least twice that of HEK sol-CD39L3-01, measured by apyrase assay.

[0012] In another aspect, the invention is directed to a nucleic acid construct comprising a nucleotide sequence encoding a signal sequence, a linker, and a soluble apyrase, wherein the linker has the sequence EVLP at its C-terminus and wherein said linker or a portion thereof may represent a sequence present in the native soluble apyrase. The invention is also directed to CHO cells containing this construct and to methods to produce EN-apyrase by culturing these cells.

[0013] In still another aspect, the invention is directed to a CHO culture system for obtaining EN-apyrase, which culture system comprises providing a medium wherein during culturing the medium maintains a glutamine concentration at about 2 mM and a pH of 7.4 and wherein the temperature of the culture is shifted from 37°C to 34°C at day 5 of culturing.

**Brief Description of the Drawings**

[0014] Figure 1 is a map of pAPT8742 for expression of sol-CD39L3 R67G T69R.

[0015] Figure 2 depicts the expression retrovector construct pCS-APT-WPRE (new ori).

[0016] Figure 3 depicts viable cell density in PFCHO LS medium of various apyrase-producing clones.

[0017] Figure 4 depicts cell viability in PFCHO LS medium of various apyrase-producing clones.

[0018] Figure 5 depicts expression levels in PFCHO LS medium of various apyrase-producing clones.

[0019] Figure 6 depicts viable cell density in OptiCHO™ medium of various apyrase-producing clones.

[0020] Figure 7 depicts cell viability in OptiCHO™ medium of various apyrase-producing clones.

[0021] Figure 8 depicts expression levels in PFCHO LS.

[0022] Figure 9 shows the stability of EN-apyrase production over 25 generations of clone 350.
Figures 10A and 10B show the isoelectric points of HEK-sol-CD39L3-01 and EN-apyrase.

Figure 11 shows the progress of purification protocol of EN-apyrase using two ion exchange chromatography steps.

Figure 12 shows the results of the determination of \textit{in vivo} half-life of HEK-sol-CD39L3-01 in rabbits.

Figure 13 shows the half-life of HEK-sol-CD39L3-01 in rabbits as compared to half-life of EN-apyrase.

Figure 14 depicts the results of Figure 13 using an \textit{ex vivo} test for ability to inhibit platelet aggregation.

Figure 15 shows results comparable to those in Figure 13 but in pigs.

Figure 16 shows results comparable to those of Figure 14 but in pigs.

**Modes of Carrying Out the Invention**

Applicants have found that by employing specifically designed recombinant materials and methods, preparations of EN-apyrase can be obtained that have superior pharmacokinetic properties, putatively due to enhanced glycosylation. The resultant EN-apyrases have lower isoelectric points, homogeneous N-termini and enhanced glycosylation, as well as longer \textit{in vivo} half-lives.

The EN-apyrase of the invention is a soluble CD39L3 or a homolog thereof. Soluble CD39L3 is represented by SEQ ID NO:1, absent at least about 43 amino acids from the N-terminus and at least about 44 amino acids from the C-terminus corresponding to the membrane spanning domains. The apyrase may be a soluble CD39L3 or an ADPase enhanced apyrase as taught by Jeong, et al. (U.S. 7,390,485), \textit{i.e.}, which contains a substitution of an arginine for a glycine at residue 67 and/or a threonine for an arginine at residue 69 (where the residue number refers to the CD39L3 SEQ ID NO:1).

A "homolog" of soluble CD39L3 includes sequences having 1 to 5 conservative substitutions, which retain ADP'ase and ATP'ase activity and/or are of 80% or 90% or 95% identical to positions 49-485 of SEQ ID NO:1. In one embodiment, the homologs comprise tandem proline residues corresponding to residues 52 and 53 of CD39L3 SEQ ID NO:1.

Exemplary soluble CD39L3 apyrase homologs comprise amino acid residues of 49-485 of SEQ ID NO:1 with the following substitutions, referring to SEQ ID NO:1: R67G T69R; T69R; R67G; R69A T69R; R67A T69H; R67A P69K; R67G T69H; R69G T69K; T69H; T69K; and R69A. The homologs will include a portion of SEQ ID NO:1 or
modifications as set forth above that include sequences beginning with the PPG residues starting at position 52.

[0034] The nucleotide sequence encoding CD39L3 is shown as SEQ ID NO:2. The amino acid sequence of soluble CD39L3 is shown in SEQ ID NO:3 and its encoding nucleotide sequence in SEQ ID NO:4. The amino acid sequence of soluble CD39L3 which is the T67G T69R mutant is shown in SEQ ID NO:5 and its coding sequence in SEQ ID NO:6. The amino acid sequence of the protein encoded by the construct used to prepare HEK-SOL-CD39L3-01, a non-enhanced form of apyrase is shown in SEQ ID NO:7 and its corresponding encoding nucleotide sequence in SEQ ID NO:8. The amino acid sequence encoded by the construct that produces one embodiment of EN-apyrase is shown in SEQ ID NO:9 and its encoding nucleotide sequence in SEQ ID NO:10.

**Secretory Signal Sequence**

[0035] The EN-apyrase of the invention are produced in a form that is secreted in to the medium, and thus the constructs for their production include a signal sequence.

[0036] The signal sequence of the present invention can be any signal sequence known to result in secretion of a protein in appropriate cell systems. Moreover, in silico methods exist to identify and predict sequences function as secretory signal sequences, for example as described by Otsuki, et al., DNA Research (2005) 12:117-126, "Signal Sequence and Keyword Trap in silico for Selection of Full-Length Human cDNAs Encoding Secretion or Membrane Proteins from Oligo-Capped cDNA Libraries."

[0037] By way of example, secretory signal sequences can be any of the sequences set forth in Table 1.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Entry Name</th>
<th>Protein Name</th>
<th>Organism</th>
<th>Length</th>
<th>Signal Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P01892</td>
<td>1A02_HUMAN</td>
<td>HLA class I histocompatibility antigen, A-2 alpha chain</td>
<td>Homo sapiens</td>
<td>24</td>
<td>MAVMAPRTLVLSSG ALALT...</td>
</tr>
<tr>
<td>P23795</td>
<td>ACES_BOVIN</td>
<td>Acetylcholinesterase</td>
<td>Bos taurus</td>
<td>30</td>
<td>MRPPWCPLHTPSLTPPLLL...</td>
</tr>
<tr>
<td>Q9GLN7</td>
<td>ACE_PANTR</td>
<td>Angiotensin-converting enzyme, somatic isoform</td>
<td>Pan troglodytes</td>
<td>27</td>
<td>MGAASGRRGPGLLLLLL...</td>
</tr>
<tr>
<td>Q28483</td>
<td>ADAM5_MACFA</td>
<td>Disintegrin and metalloproteinase domain-containing protein 5</td>
<td>Macaca fascicularis</td>
<td>16</td>
<td>MFLLLVLLTLGGMHA</td>
</tr>
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<td>Organism</td>
<td>Length</td>
<td>Signal Sequence</td>
</tr>
<tr>
<td>------------------</td>
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<td>----------------------------------</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>A2AJA7</td>
<td>AEGP_MOUSE</td>
<td>Apical endosomal glycoprotein</td>
<td>Mus musculus</td>
<td>21</td>
<td>MCLPSHLLSTWVLFMAAQSL...</td>
</tr>
<tr>
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<td>AMYP_MOUSE</td>
<td>Pancreatic alpha-amylase</td>
<td>Mus musculus</td>
<td>15</td>
<td>MKFVLLLLSLIGFCWA</td>
</tr>
<tr>
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<td>Cadherin-2</td>
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</tr>
<tr>
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<td>Cadherin-8</td>
<td>Rattus norvegicus</td>
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</tr>
<tr>
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<td>Macaca mulatta</td>
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<td>MKVSTTLWLPLLAAAFSPQ...</td>
</tr>
<tr>
<td>Q8HYP4</td>
<td>CCL23_MACMU</td>
<td>C-C motif chemokine 23</td>
<td>Macaca mulatta</td>
<td>21</td>
<td>MKVSAALSCLMLVTAALGSQ...</td>
</tr>
<tr>
<td>P28325</td>
<td>CYTD_HUMAN</td>
<td>Cystatin-D</td>
<td>Homo sapiens</td>
<td>20</td>
<td>MMWPMHTPLLLLTLALMVAVA</td>
</tr>
</tbody>
</table>

**Linker Moiety**

[0038] It has been found that by providing a sequence at a desired N-terminus of an apyrase having the sequence EVLP, cleavage may be effected in Chinese Hamster Ovary (CHO) cells such that a uniform N-terminus is produced immediately upstream of the glutamic acid residue represented by E. However, in this sequence, the glutamic acid residue may be replaced by aspartic (D) glutamine (Q) or asparagine (N). The linkages represented in these sequences are resistant to proteases in CHO cells.

[0039] It should be noted that the linker sequence may in fact be part of the apyrase sequence, as is the case in the constructs illustrated in the examples below. Thus, the EVLP sequence becomes the N-terminus of the EN-apyrase produced. This is within the amino acid sequence of the apyrase encoded by the illustrative constructs.

[0040] Regardless of the amino acid sequence of the soluble apyrase, including the linker sequence with the carboxyl terminus as described above ensures cleavage upstream of the E, D, Q or N residue when the apyrase is secreted into the culture medium. Additional sequence downstream of the signal sequence may be present upstream of the E, D, Q or N residue, including 0-10 amino acids, preferably 1-5 amino acids.

[0041] Thus, upon proper pairing with the signal sequence and linker moiety, an apyrase construct, when transformed into CHO cells, produces a translation product with a single, strong signal peptidase cleavage site, and secretes an apyrase with a homogeneous N-terminus. "Homogeneous" includes "substantially homogeneous", *e.g.*, more than any of about 80% or 90%, 95% or 99% of the EN-apyrase molecules processed to have the same N-terminus.
The EN-apyrases comprise substantially more glycosylation than the apyrase produced by HEK cells transformed with a construct encoding SEQ ID NO:7 (i.e., HEK-sol-CD39L3-01 as described in Preparation A). EN-apyrases are produced and secreted in mammalian cell culture with a pi in a range of about 3.0 to about 4.5. Without being bound by theory, applicants believe that the N-terminal amino acid sequence modifications cause altered N-terminus endopeptidase processing, resulting in conformational changes sufficient to alter glycosylation. Moreover, a combination of conformational changes and glycosylation are responsible for the unexpected pharmacokinetic properties of EN-apyrase. The reduced isoelectric point is believed due to an increased sialic acid content in the increased glycosylation.

One of the superior, unexpected properties of the EN-apyrases of the present invention is a glycoprotein product that can readily be purified by ion exchange chromatography. As shown below, EN-apyrase can be purified to about 90% or higher in a two step ion exchange protocol.

The instant EN-apyrases have an extended circulating half-life, in comparison to HEK-sol-CD39L3-01 as shown below. EN-apyrases have a T½ in rabbits or pigs of at least about 2X or at least about 4X or of at least about 5X or of at least about 8X that of HEK-sol-CD39L3-01. Such increased half-life is especially useful for therapeutic agents such as EN-apyrases that are typically administered parenterally.

Methods for Production of EN-apyrases

The EN-apyrases of the invention may be produced in CHO cells under control of a signal sequence as set forth above, and under culture conditions that result in these enhanced properties. Optimally, these conditions include maintaining a glutamine content in the medium of about 2 mM, maintaining the pH at about 7.4 and altering the temperature from 37°C to 34°C after 5 days of culture. Variations in these parameters are permitted, but for optimal production of the EN-apyrases, these conditions are reliably successful. For example, the glutamine content should be maintained between about 1.5 mM and 4 mM, preferably 2-3 mM. The pH should be maintained between about 7.0 and 7.8, preferably between 7.2 and 7.6. The temperature should be lowered to between about 31°C-36°C, preferably 33°C-35°C. This can be done between 4 and 6 days after the start of culture.
Uses

[0046] The instant EN-apyrases are therapeutic agents useful for at least the uses of apyrases generally, CD39 compounds more specifically, and any of CD39L1-8, e.g., CD39L3 compounds. EN-apyrases are useful as anti-platelet, anti-thrombolytic agents and as anti-inflammatory and endothelial cell (EC) protective proteins. Additionally, EN-apyrases are therapeutically useful for conditions taught in U.S. provisional application 61/294,725 filed 13 January 2010 entitled "Apyrase Therapy for Bleeding Conditions", incorporated by reference in its entirety. Conditions that can usefully be treated by EN-apyrases include conditions of bleeding from injury caused by mechanical or pharmacologic insult.

[0047] In some biologic conditions, EN-apyrases of the present invention serve a therapeutic role in a plurality of functions. For example, the anti-inflammation and anti-thrombosis function of EN-apyrases results in an unexpected therapeutic efficacy in various conditions.


[0050] Certain clinical situations may require the slow and prolonged release of biologically active EN-apyrases or biological derivatives. Such situations may require the sequestrations of EN-apyrases or biological derivatives in, for example, hydrogel or other pharmaceutically
acceptable polymerizable gels. Additionally, a polyethylene glycol (PEG) can be added to prolong the blood half-life to increase efficacy of a soluble EN-apyrases. In the case where EN-apyrases are used as a preventative medication, this may allow for single-bolus dose administration to maintain protective effects of EN-apyrases for longer periods. Other protein modifications to alter protein half-life include, for example, albumin conjugation, IgG fusion molecules and altering of the proteins glycosylation pattern.

[0051] It is also envisioned in the present invention that certain medical procedures or instances may require inhibition of circulating Instant apyrase activity. Such inhibitors could be, for example, pharmaceutically acceptable enzyme inhibitors (for example, ADP analogues), pharmaceutically acceptable calcium chelators, antibodies specific to Instant apyrase. Other medical procedures could also include, for example, blood transfusions or platelet transfusions.

[0052] EN-apyrases and biologically active derivatives are useful in any clinical situation where the hydrolysis of ATP and/or ADP to AMP is clinically beneficial including disease states where ATP and/or ADP concentrations are abnormally high. EN-apyrases and biologically active derivatives are beneficial in clinical situations where platelets or activated platelets play an important role in disease progression, for example, tumor metastases (Bakewell, S. J., *et al*, *PNAS* (2003) 100:14205-14210).

[0053] The clinical and biological effectiveness of the administered EN-apyrases or biological derivative can be readily evaluated at given time intervals after administration. For example, administration of EN-apyrases or biological derivatives should promote longer bleeding times in the setting where platelet count remains unchanged. Additionally, direct measurement of blood samples for enzyme activity of EN-apyrases or metabolites will also indicate presence of the molecule in the circulating blood. Based on precise sampling of blood samples coupled with methods known in the art for assessing biochemical function of EN-apyrases the half life of the protein can be estimated. Additional clinically relevant assays for the presence of biologically active EN-apyrases or biologically active derivative may also be envisioned.

Methods for *In Vitro* and *In Vivo* Validation of Instant Apyrase Efficacy

[0054] Biochemical function of EN-apyrases may be assessed by numerous methods available to one skilled in the art. For example, ATP'ase and ADP'ase enzymatic activities of purified EN-apyrases can be determined at 37°C in a 1 ml solution containing 8 mM CaCl2, 200 µM substrate (ATP for ATP'ase or ADP for ADP'ase), 50 mM imidazole, and 50 mM Tris, pH 7.5 (Picher, *et al*, *Biochem. Pharmacol.* (1988) 51:1453). The reaction can be stopped and
inorganic phosphate released can be measured by addition of 0.25 ml of malachite green reagent (Baykov, et al., Anal. Biochem. (1988) 171:266). Based on the spectrophotometry analysis at 630 nm, one unit of ATP'ase (or ADP'ase) corresponds to release of 1μ mole of inorganic phosphate/min at 37°C. Key kinetic constants for the enzyme such as Kₘ and k cat may be obtained by fitting data into, for example, a Michaelis-Menten equation. Other assays useful for monitoring biochemical function include, but are not limited to, a radiometric assay, a HPLC assay both described by Gayle III, et al. (J. Clin Invest. (1998) 101:1851-1859) or a radio-TLC assay described by Marcus, A. J., et al. (J. Clin Invest. (1991) 88:1690-1696).


Therapeutic Compositions of EN-apyrases

[0057] The present invention provides compositions comprising a biologically effective amount of EN-apyrase or biologically active derivative in a pharmaceutically acceptable dosage. Therapeutic composition of EN-apyrases or biologically active derivative may be administered clinically to a patient before symptoms, during symptoms, or after symptoms. After symptom administration of EN-apyrases or biologically active derivatives may occur, for example, between 0 and 48 hours after the onset of stroke. Administration of EN-apyrases or biologically active derivatives to achieve therapeutic effect may be given by, for example, bolus
injection, intramuscularly, subcutaneously, inhalation, continuous infusion, sustained release, or other pharmaceutically acceptable techniques. Certain clinical situations may require administration of EN-apyrases or biologically active derivatives as a single effective dose, or may be administered daily for up to a week or a much as a month or more. Ideally EN-apyrases will be administered to patients in a pharmaceutically acceptable form containing physiologically acceptable carriers, excipients or diluents. Such diluents and excipients may be comprised of neutral buffered saline solution, antioxidants (for example ascorbic acid), low molecular weight polypeptides (for example polypeptides \( \leq 10 \) amino acids) amino acids, carbohydrates (for example, glucose, dextrose, sucrose, or dextran), chelating agents such as EDTA, stabilizers (such as glutathione). Additionally, cosubstrates for the EN-apyrases or biologically active derivatives, for example, calcium (Ca 2+) may be administered at time of dosage for maximal activity of the enzyme. Such carriers and diluents are selected to be nontoxic to the patient at recommended dosages and concentrations. It is also envisioned in the present invention that EN-apyrases or biologically active derivatives may be administer with other agents that synergistically enhance the benefit of EN-apyrases or biologically active derivatives alone. For example, it is envisioned that administration of other antiplatelets or anticoagulants, such as aspirin, heparin or bivalirudin with EN-apyrases or biologically active derivative may have additional benefits such as improve reperfusion, extend therapeutic time window, prevent reocclusion, and prevent microvascular thrombosis. It is also envisioned that administration of EN-apyrases or biologically active derivatives may improve efficacy and lower the effective dosage of thrombolytics (Activase®, TNKase™, vampire bat plasminogen activator, urokinase, streptokinase, staphylokinase, and ancrod). It is still further envisioned in the present invention that operable fusion polypeptides between, for example, and ADP enhanced apyrase and thrombolytic (for example, TNKase) may provide an ideal therapeutic solution for acute myocardial infarction (AMI), percutaneous coronary intervention (PCI) and acute ischemic stroke (AIS).

[0058] Dosage requirements of EN-apyrases or biologically active derivatives may vary significantly depending on age, race, weight, height, gender, duration of treatment, methods of administration, biological activity of EN-apyrases, and severity of condition or other clinical variables. Effective dosages may be determined by a skilled physician or other skilled medical personnel.

[0059] The citations provided herein are hereby incorporated by reference for the cited subject matter.
Preparation A
Production of sol CD39L3 in Human Kidney Cell-Derived HEK293T Cells

[0060] HEK 293T cell lines were stably transformed with a construct that provides for the expression of SEQ ID NO:7. This sequence shows soluble CD39L3 R676 T69R mutant coupled to an underlined signal sequence from mouse IgG kappa. This sequence was inserted to provide the plasmid pAPT8742 shown in Figure 1. The secreted protein product designated here as "HEK-sol-CD39L3-01".

[0061] Transformants were adapted to serum-free suspension culture and continually split to larger flasks until a 3L spinner was inoculated. The cells were split every 3 - 4 days and apyrase in conditioned medium was collected. To produce sufficient quantity of the protein for preclinical validation and determine viability of apyrase manufacturing in a commercial scale, a pilot study was carried out in 30L bioreactors. A typical 30L bioreactor was inoculated at 0.5x10^6 cells per mL and in 5 to 6 days HEK 293T cells grew typically over 3.5x10^6 cells per mL and produced 2-3 mg of apyrase/L. A purification process was developed involving DEAE, size exclusion and heparin affinity columns, which leads to 30% recovery yield of apyrase.

[0062] Conditioned medium harvested from the culture of HEK293T cells transformed loaded on to a DEAE column equilibrated with 10 mM Tris-HCl, pH 7.4 after the buffer exchanged to the equilibration buffer. The apyrase fraction was eluted with 10 mM Tris-HCl, pH 7.4/100 mM NaCl. After another buffer exchange to the equilibration buffer the eluted fraction was loaded on a Heparin column equilibrated with 10 mM Tris-HCL, pH 7.4. The apyrase was eluted with 10 mM Tris-HCl, pH 7.4/30 mM NaCl and concentrated using Amicon stirred cell concentrator (Millipore).

[0063] N-terminal analysis of the purified apyrase was performed by Edman degradation and showed three different N-terminal deletions. The predicted cleavage from signal would result in an N-terminal asp shown as position 21 of SEQ ID NO:7. The recovered protein was 27% N-terminal lys (position 30), 40% N-terminal glu (position 32) and 33% N-terminal val (position 33).

Example 1
Constructs for Production of an Enhanced Apyrase

[0064] An apyrase construct was designed to encode an EN-apyrase based upon sol-CD39L3 R67G T69R. The signal sequence was the bovine a-lactalbumin signal peptide. The apyrase moiety starts at residue 49 of SEQ ID NO:1, and the encoded signal — apyrase fusion is shown in SEQ ID NO:9.
[0065] The sequence of the EN-apyrase construct inserted in the final expression retrovector plasmid is shown in Table 2 below. This construct was inserted into a retroviral vector and is designated "APT" in the resulting vector shown in Figure 2.

[0066] The position of MfeI and XhoI restriction sites used for cloning the DNA fragment into the host plasmid are shown. The first 19 codons encode the signal peptide. During DNA sequencing of the final construct, a silent mutation was detected at position 2879 (AAC instead of the predicted AAT). This codon is double underlined. The final vector was designated oCS-APT-WPRE (new ori) (Figure 2).

Table 2

MfeI

2491 TCGAAAGCTT CTA GACAATT GCGGCCACC ATG ATG TCC TTT GTC TCT CTG 2540
1 Met Met Ser Phe Val Ser Leu 7
a-Lactalbumin Signal Peptide

2541 CTC CTG GTT GGC ATC CTA TCC CAT GCC ACC CAG GCC GAG GTC CTC 2585
8 Leu Leu Val Gly Ile Leu Phe His Ala Thr Gin Ala Glu Val Leu 22

| 2586 CCT CCA GGA CTG AAG TAT GGT ATT GTG CTG CAT GCC GGG TCT TCA 2620 |
|---|---|
| 23 Pro Pro Gly Leu Lys Tyr Gly H e Val Leu Asp Ala Gly Ser Ser 37 |

| 2631 GGG ACC CGC GTC TAC GTG TAT CAA TGG CCA GCA GAA AAA GAG GTT 2675 |
|---|---|
| 38 Gly Thr Arg Val Tyr Val Tyr Gin Trp Pro Ala Glu Lys Glu Asn 52 |

| 2676 AAT ACC GGA GTG GTC AGT CAA ACC TCC AAA TGT AGT GTG AAA GGC 2720 |
|---|---|
| 53 Asn Thr Gly Val Val Ser Gin Thr Phe Lys Cys Ser Val Lys Gly 67 |

| 2721 TCT GGA ATC TCC AGC TAT GGA AAT AAC CCC CAA GAT GTC CCC AGA 2765 |
|---|---|
| 68 Ser Gly Ile Ser Tyr Gly Asn Asn Pro Gin Asp Val Pro Arg 82 |

| 2766 GCC TTT GAG GAG TGT ATG CAA AAA GTC AAG GGG CAG GTT CCA TCC 2810 |
|---|---|
| 83 Ala Phe Glu Glu Cys Met Gin Lys Val Lys Gly Gin Val Pro Ser 97 |

| 2811 CAC CTC CAC GGA TCC ACC CCC ATT CAC CTG GGA GCC ACG GCT GGG 2855 |
|---|---|
| 98 His Leu His Gly Ser Thr Pro H e His Leu Gly Ala Thr Ala Gly 112 |

| 2856 ATG CGC TTG CTG AGG CAA ACC AAC ACC GCA ACC AAC ACC AAT GAA GCT 2900 |
|---|---|
| 113 Met Arg Leu Leu Arg Leu Gin Asn Thr Ala Ala Asn Glu Val 127 |

| 2901 CTT GAA AGC ATC CAA AGC TAC TTT AGC TCC CAG CCC TTT CAG TCT 2945 |
|---|---|
| 128 Leu Glu Ser Ile Gin Ser Tyr Phe Lys Ser Gin Pro Phe Asp Phe 142 |

| 2946 AGG GGT GCT CAA ATC ATT TCT GGG CAA GAA GGA GGG GTA TAT GGA 2990 |
|---|---|
| 143 Arg Gly Ala Gin H e H e Ser Gly Gin Glu Glu Glu Val Tyr Gly 157 |

| 2991 TGG ATT ACA GCC AAC TAT TTA ATG GGA AAT TCC CTG GAG AAG AAC 3035 |
|---|---|
| 158 Trp Ile Thr Ala Asn Tyr Leu Met Gly Asn Phe Leu Glu Lys Asn 172 |

| 3036 CTG TGG CAC ATG TGG GTG CAC CCG CAT GGA GTG GAA ACC ACG GTT 3080 |
|---|---|
| 173 Leu Trp His Met Trp Val His Pro His Gly Val Glu Thr Thr Gly 187 |

| 3081 GCC CTG GAC CTA GGT GGT GCC TCC ACC CAA ATA TCC TTC GTG GCA 3125 |
|---|---|
| 173 Leu Trp His Met Trp Val His Pro His Gly Val Glu Thr Thr Gly 187 |
188 Ala Leu Asp Leu Gly Gly Ala Ser Thr Gin H e Ser Phe Val Ala 202
3126 GGA GAG AAG ATG GAT CTG AAC ACC AGC GAC ATC ATG CAG GTG TCC 3170
203 Gly Glu Lys Met Asp Leu Asn Thr Ser Asp H e Met Gin Val Ser 217
3171 CTG TAT GCC TAC GGA TAC ACC ATG AAC ACC AGC GAC ATC ATG 3215
218 Leu Tyr Gly Tyr Val Tyr Thr Leu Tyr Thr His Ser Phe Gin Cys 232
3216 TAT GGC CGG AAT GCT GAG AAG AGG AAG ATG GTC GCA ATG TCG 3260
233 Tyr Gly Arg Asn Glu Ala Glu Lys Phe Leu Ala Met Leu Leu 247
3261 CAG AAT TCT CCT ACC AAA AAC CAT CTC ACC AAT CCC TGT TAC CCT 3305
248 Gin Asn Ser Pro Thr Lys Asn His Leu Thr Asn Pro Cys Tyr Pro 262
3306 CGG GAT TAT AGC ATG TCC ACC ATG GGC CAT GTA TTT GAT AGC 3350
263 Arg Asp Tyr Ser Ile Ser Phe Thr Met Gly His Val Phe Asp Ser 277
3351 CTG TGC ACT GTG GAC CAG AGG CCA GAA AGT TAT AAC ACC AAT 3395
278 Leu Cys Thr Val Asp Gin Arg Pro Glu Ser Tyr Asn Pro Asn Asp 292
3396 GTG ACT GCT TTT GAA GGA ACT GGG GAC CCA TCT GTG TGT AAG GAG 3440
293 Val Ile Thr Phe Glu Gly Thr Gly Asp Pro Ser Leu Cys Lys Glu 307
3441 AAG GTG GCT TCC ATA TTT GAC TTC AAA GCT TGC CAT GAT CAA GAA 3485
308 Lys Val Ala Ser H e Phe Asp Phe Lys Ala Cys His Asp Gin Glu 322
3486 ACC TGT TCT TTT GAT GGG GTT TAT CAG CCA AAG ATT AAA GGC CCA 3530
323 Thr Cys Ser Phe Asp Gly Val Tyr Gin Pro Lys H e Lys Gly Pro 337
3531 TTT GTG GCT TTT GCA GGA TTC TAG TAC ACA GCC AGT GCT TTA AAT 3575
338 Phe Val Ala Phe Ala Gly Phe Tyr Tyr Thr Ala Ser Ala Leu Asn 352
3576 CTT TCA GGT AGC TTT TCC CTG GAC ACC TTC AAC TCC AGC TAC TGG 3620
353 Leu Ser Gly Ser Phe Ser Leu Asp Thr Phe Asn Ser Ser Thr Trp 367
3621 AAT TTC TGC TCA CAG AAT TGG AGT CAG CTC CCA CTG CTG CTC CCC 3665
368 Asn Phe Cys Ser Gin Asn Trp Ser Gin Leu Pro Leu Leu Leu Pro 382
3666 AAA TTT GAT GAG GTA TAT GCC CGG TCT TAC TGC TCC TCA GCC AAC 3710
383 Lys Phe Asp Glu Val Tyr Ala Arg Ser Tyr Cys Phe Ser Ala Asn 397
3711 TAC ATC TAC CAC TTT CGC TAG TAC AAA TTC ACA GCC GAG GAG 3755
3711 TAC ATC TAC CAC TTT CGC TAG TAC AAA TTC ACA GCC GAG GAG 3755
398 Tyr Ile Tyr His Leu Phe Val Asn Gly Tyr Lys Phe Thr Glu 412
3756 ACT TGG CCC CAA ATA CAC TTT GAA AAA GAA GTG GGG AAT AGC AGC 3800
413 Thr Trp Pro Gin H e His Phe Glu Lys Glu Val Gly Asn Ser Ser 427
3801 ATA GCC TGG TCT CTT GCC TAC ATG CTC AGC CTG ACC AAC CAG ATC 3845
428 Ile Ala Trp Ser Leu Gly Tyr Met Leu Ser Leu Thr Asn Gin H e 442
3846 CCA GCT GAA AGC CCT CTG ATC CTG ACC CCC ATA GAA CCA CCT GCT 3890
443 Pro Ala Glu Ser Pro Leu H e Arg Leu Pro H e Glu Pro Pro Val 457

xho f
Cla I

3891 TGA TGAGATC TCGAGTTCGA CATCGATAAT CAACCTCT GG ATTACAAAAAT 3940
458 TRM 458
Example 2
Transformation of CHO cells with oCS-APT-WPRE (new ori)

[0067] Chinese Hamster Ovary (CHO) production cell lines were made by two rounds of transduction of the CHO parental cell line with retrovector constructed in Example 1. The pooled population of transduced cells was named sCHO-S/sC-APT-R 2X. Samples of the pooled population cell lines were cryopreserved after each transduction. The pooled population of sCHO-S/sC-APT-R 2X cells was diluted to very low cell density (approximately 0.5 or 0.75 viable cells per 200 µL media) and plated in 96 well microtiter plates to establish clonal cell lines that originated from single cells. A total of 560 clones were screened for EN-apyrase production by Malachite Green assay after 14 days of incubation. Twenty four of the top clones based on EN-apyrase production were expanded from 96 well plates to 24 well plates. Twenty of the 24 clones survived expansion and were cryopreserved.

[0068] The 20 clones were screened in triplicate T175 flasks for productivity. The top 5 clones chosen were clones 176, 248, 290, 350 and 372. Selected clonal lines of sCHO-S/sC-APT-R 2X cells were tested for replication competent retrovirus (RCR), mycoplasma contamination and bioburden with negative results.

[0069] The cell lines prepared above were passaged every 3-4 days during exponential phase for all of the studies outlined below, maintaining a viability of 90% or better in both PFCHO LS (HyClone) and CD OptiCHO™ (Invitrogen) media. Cells were inoculated at a cell density of 300,000 cells/ml in each medium and incubated in a Multitron shaking incubator at a speed of 150 rpm. The initial temperature set point was 37°C. The temperature was shifted to 31°C on Day 5 under Conditions 2 and 4. The CO₂ set point was 5% and the Feed Supplement: was HyClone supplements R15.4 and PS307 and glutamine.

[0070] Four different conditions, as outlined below, were performed in duplicate 125 mL shake flask (50 mL total volume) for each clone. There were only enough cells of clone #248 in PFCHO LS to be tested under Conditions 1 and 2. Shakers were harvested on Day 14.

Condition 1

Day 0: 3 g/L PS307
Day 2: 3g/L PS307 + 3 mM glutamine
Day 4: 3 g/L R15.4 + 3 mM glutamine
Day 6: 12 g/L R15.4
Condition 2

Day 0: 3 g/L PS307
Day 2: 3 g/L PS307 + 3 niM glutamine
Day 4: 3 g/L R 15.4 + 3 niM glutamine
Day 5: Temperature shift to 31°C
Day 6: 12 g/L R 15.4

Condition 3

Day 0: 1.5 g/L PS307 + 1.5 g/L R 15.4
Day 2: 1.5 g/L PS307 + 1.5 g/L R 15.4 + 3 mM glutamine
Day 4: 1.5 g/L PS307 + 1.5 g/L R 15.4 + 3 mM glutamine
Day 6: 5 g/L PS307 + 5 g/L R 15.4

Condition 4

Day 0: 1.5 g/L PS307 + 1.5 g/L R 15.4
Day 2: 1.5 g/L PS307 + 1.5 g/L R 15.4 + 3 mM glutamine
Day 4: 1.5 g/L PS307 + 1.5 g/L R 15.4 + 3 mM glutamine
Day 5: Temperature shift to 31°C
Day 6: 5 g/L PS307 + 5 g/L R 15.4

[0071] The results from the various conditions and media are shown in Figure 3 through Figure 8 below. The cell densities peaked at 60 x 10^5 cells/mL in the PFCHO LS medium (Clone #176) and 160 x 10^5 cells/mL in the OptiCHO™ medium (Clone #176).

[0072] The results demonstrate various useful conditions for culturing the CHO cells for production of EN-apyrase. These results also indicate that the productivity was not directly related to cell density.

[0073] The addition of a temperature shift in the OptiCHO™ cultures generated a significant increase in the overall titer which led to the selection of either condition 2 or 4 in OptiCHO™ as the preferred method of culturing Clone #350.

Example 3

Improved Cell Culture Conditions to Increase Protein Yield in 10 L Bioreactors

[0074] Background: Preliminary studies show that PowerCHO®-2 medium resulted in higher yield and better glycosylation of EN-apyrase in shaking flasks. Two different conditions
were run in 10 L bioreactors (Vessel 1 and 2) in which the pH was maintained at 7.4. An additional 10 L bioreactor (Vessel 3) was run under the same condition as Vessel 2 but with a natural drift of the pH down to 7.0.

[0075] Materials and Methods: Cell line CHO-S-APT-R Clone # 350 was passaged every 3-4 days during the exponential growth phase for scale-up for the 10 L Braun bioreactors. Cells were inoculated at a cell density of approximately 300,000-400,000 cells/ml in PowerCHO®-2 (Lonza) medium into three 10 L bioreactors. Fed-batch supplements used for this study were HyClone PS307 (12% (w/v) solutions), AGT CD CHO 5X Feed Medium Complete (Invitrogen), AGT CD CHO 5X Feed Medium Complete + 12.5 g/L galactose (Invitrogen), 45% glucose solution, 20% glucose/galactose solution, 200 mM L-glutamine, 50X solution of L-Asparagine (15 g/L)/L-Serine (10 g/L), 50X solution of L-Tyrosine (4 g/L)/L-Cystine (2 g/L).

Condition 1

DO: 3 g/L PS307 + 2 mM glutamine
D1: Maintain glutamine to 2 mM; Maintain glucose to 5 g/L
D2: 3 g/L PS307 + glucose (to 5 g/L) + glutamine (to 2 mM)
D3: Maintain glutamine to 2 mM; Maintain glucose to 5 g/L
D4: 3 g/L R15.4/PS307 (1:1) + glutamine (to 2 mM) + glucose (to 5 g/L)
D5: Maintain glutamine to 2 mM; Maintain glucose to 5 g/L
D5: Temperature shift to 34°C
D6: 12 g/L R15.4
D6: Maintain glutamine to 2 mM; Maintain glucose to 4 g/L
D7-D16: Maintain glutamine to 2 mM; Maintain glucose to 4 g/L
pH 7.4 throughout the run
Condition 2

DO: 3 g/L PS307

Dl: Maintain glutamine to 2 mM

D3: 30% v/v (AGT CD 5X Medium Complete + 12.5 g/L galactose) + IX L-Asparagine (0.3 g/L)/L-Serine (0.2 g/L) + IX L-Tyrosine (80 mg/L)/L-Cystine (40 mg/L) + 3 mM Glutamine if VCD ≥ 10 x 105 cells/ml on Day 2 or Day 3 by default

D4-D5; Maintain glutamine to 2 mM

Day 5: Temperature shift to 34°C

D6-D16: Feed 10% (v/v) (AGT CD 5X Feed Medium Complete + 12.5 g/L galactose) when glucose levels are ~ 5 g/L

D6-D16 Post 10% 5X Feed: Feed 3 g/L glucose/galactose when glucose levels are ~ 3 g/L

pH 7.4 throughout the run

Condition 3

DO: 3 g/L PS307

Dl: Maintain glutamine to 2 mM

D3: 30% v/v (AGT CD 5X Medium Complete + 12.5 g/L galactose) + IX L-Asparagine (0.3 g/L)/L-Serine (0.2 g/L) + IX L-Tyrosine (80 mg/L)/L-Cystine (40 mg/L) + 3 mM Glutamine

D4-D5; Maintain glutamine to 2 mM

Day 5: Temperature shift to 34°C

D6-D16: Feed 10% (v/v) (AGT CD 5X Feed Medium Complete + 12.5 g/L galactose) when glucose levels are ~ 5 g/L

D6-D16 Post 10% 5X Feed: Feed 3 g/L glucose/galactose when glucose levels are ~ 3 g/L

pH natural drift to 7.0 ± 0.05

[0076] Results: Vessel 3 had the highest peak cell density (70 x 105 cells/mL). Vessels 1 and 2 peaked at 48 x 105 cells/mL. Vessel 1 harvested at day 14, Vessel 2 at day 16 and Vessel 3 at day 15. All the vessels were < 50% viability at harvest. Maximum protein levels (activity assay) for Vessels 2 and 3 were 55 mg/L at harvest. Vessel 1 had a protein level of 51 mg/L at harvest. Glucose was not rate-limiting for any of the vessels. Glutamine levels were low at the end of the runs for Vessels 2 and 3. Lactate levels were high for Vessels 1 and 2
(~ 6 g/L) while vessel 3 was < 2.5 g/L. Ammonium levels stayed below 10 mM for Vessels 1 and 2 and ~ 16 mM for Vessel 3.

[0077] Conclusion: Overall, protein levels were better for conditions 2 and 3. Lactate levels were much higher in Vessels 1 and 2 which resulted in over 1 L of base added to help maintain the pH. Vessel 3 required only 200 mL of base to maintain pH throughout the entire run. The addition of the base also caused the osmolality (data not shown) to be much higher (~ 540 mmol/kg for Vessel 1 and ~ 500 mmol/kg for Vessel 2) than Vessel 3 (~ 365 mmol/kg). From the protein analysis, protein quality does not appear to differ between the three conditions used in this study.

Example 4
Stability of Transformed CHO cells expressing EN-apyrase

[0078] A CHO cell line produced as in Example 2 was thawed and cultured in 125 ml shaker flasks using CD OptiCHO™ media. Since the CHO cells were previously grown in PF CHO LS media, the shaker flask was carried through five passages to adapt the culture to the new media. The cells after adaptation were designated generation 0 for this study. Cells were continuously cultured by serial passage. The plan for the study was to compare cells after approximately 10, 15 and 20 generations of continuous culture. At generations 9, 17 and 24, samples of cells were frozen. At the end of the culturing, samples of cells from generations 0, 9, 17 and 24 were thawed and used to conduct terminal batch culture runs to compare EN-apyrase production of the cell line at different generations in the same experiment. The terminal culture did not include feeds and temperature shifts, as done during process development. The expression level of apyrase as determined by the Malachite green ADP hydrolysis activity assay was shown to be comparable for generations 0 and 24, respectively.

[0079] To compare the stability of gene inserts, samples of CHO cells after 0, 9, 17 or 24 generations were used for DNA isolation. Using real-time PCR on genomic DNA, the number of genetic inserts remained steady in the cell line over the next 40 generations. The PCR based index of copy number for the different generations were not significantly different for the different generations.

Example 5
Stability of Protein Production in Cell Culture

[0080] The cultures of Example 2 were incubated in 125 ml shaker flasks in 50 mL of media. CD OptiCHO™ media (Gibco Cat. #12681-011, Lot 06291004, Exp. 08/30/07) medium
with 6 mM L-glutamine (HyClone Cat. #SH30034.01, Lot 06263003) was used throughout. This cell line (Passage 6) was thawed and used to initiate the culture. Since the cell line had previously been cultured in PF CHO LS media, the culture was carried through five passages in the CD OptiCHO™ media to adapt the cells to the new media. Cultures were initially seeded at a target density of 2.5 x 10^5 viable cells/mL. Cells were passaged twice a week by taking a cell count with a Cedex™ instrument (Innovatis, Germany) and diluting the culture to the target density in fresh media. The weekly cell count data for the Research Cell Bank sample are shown in Table 3.

**Table 3**

<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Cell Viability (%)</th>
<th>Cell Count (cells/ml)</th>
<th>Generation Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>81.3</td>
<td>0.46</td>
<td>Adaptation to CD OptiCHO™ media</td>
</tr>
<tr>
<td>8</td>
<td>95.4</td>
<td>4.39</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>97.5</td>
<td>70.26</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>98.6</td>
<td>23.65</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>99.1</td>
<td>40.12</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>98.9</td>
<td>32.31</td>
<td></td>
</tr>
<tr>
<td>13 Freeze</td>
<td>98.9</td>
<td>73.96</td>
<td>0.00</td>
</tr>
<tr>
<td>14</td>
<td>99.5</td>
<td>43.79</td>
<td>4.14</td>
</tr>
<tr>
<td>15 Freeze</td>
<td>98.9</td>
<td>82.33</td>
<td>9.21</td>
</tr>
<tr>
<td>16</td>
<td>98.5</td>
<td>17.58</td>
<td>12.05</td>
</tr>
<tr>
<td>17 Freeze</td>
<td>95.9</td>
<td>68.09</td>
<td>16.87</td>
</tr>
<tr>
<td>18</td>
<td>97.6</td>
<td>14.19</td>
<td>19.37</td>
</tr>
<tr>
<td>19 Freeze</td>
<td>94.3</td>
<td>73.72</td>
<td>24.32</td>
</tr>
</tbody>
</table>

Calculations:

[0081] Generation Number (G): \( G = \frac{\text{LN}(\text{total cell count})}{\text{total cell count seeded}} \) 0.69.

[0082] Direct Comparison of Generations 0, 10, 15 and 20. After approximately 0, 10, 15 or 20 generations of culturing as described above, cells from each shaker flask were frozen down in freezing medium (46.25% conditioned media, 46.25% fresh media, 7.5% DMSO (Sigma Cat #D2650, lot 46K2381). The vials were placed at -80°C for 1-7 days and then transferred to liquid nitrogen for storage.

[0083] Upon completion of the culturing for 20 generations, frozen cells from the starting cell line after adaptation to CD OptiCHO™ media (generation 0), generation 9, generation 17 and generation 24 vials were thawed. Each of the samples was inoculated into triplicate 125 mL shaker flasks starting with approximately 250,000 cells/mL, using CD OptiCHO™ medium.
Cultures were grown at 37°C in a 5% CO₂ atmosphere shaking at 140 rpm. Samples were collected on day 14 of culture for protein analysis. Levels of production of apyrase were estimated using the Malachite green activity assay previously described.

Results.

[0084] Terminal Culture with Cells at Generations 0, 9, 17 and 24. The results below show the analysis of samples for apyrase expression during the side by side comparison of the starting cell line (research cell bank at generation 0 after adaptation to CD OptiCHO™ media) and cell lines after 9, 17 and 24 generations. Each generation sample was cultured in triplicate and the results were averaged. The error bars show the standard deviation of the samples for each generation. Figure 9 shows the average day 14-protein production for each of the four generation levels.

[0085] SDS PAGE Analysis. Samples of media containing EN-apyrase protein were collected from day 14 of productivity tests. The proteins were separated on polyacrylamide gel electrophoresis. Briefly, media samples (20 µL) were prepared in reducing sample buffer and heated at 70°C for 5 min. Size standards were SeeBlue® Plus 2 protein standards. (Invitrogen Cat #LC5925). Samples were loaded onto a 4-12% Bis-Tris NuPage® gel (Invitrogen, Cat. #NP0321) and electrophoresed at 200V for 60 minutes. The gel was stained with Gelcode™ Blue stain (Pierce Cat. #24592) at room temperature for 45 minutes. Production of EN-apyrase remained constant over 24 generations.

[0086] The stability of gene inserts from cells of various generations was compared using a gene copy index assay.

[0087] A sample of the EN-apyrase producing CHO cell line, Clone # 350, from the research cell bank after adaptation to CD OptiCHO™ media was used to isolate genomic DNA for a generation 0 sample. Samples of the generation 9, 17 and 24 EN-apyrase producing CHO cell lines for clone #350, were also used to isolate genomic DNA. The gene copy index of the EPR relative to the control β 1, 4 galactosyltransferase was measured using real time PCR. The results shown below demonstrate that there is no significant change in the gene copy index values over the 20 generations. This indicates that the number of transgene inserts does not change over extended passage of the cell line.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Gene index</th>
<th>Std. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.67</td>
<td>0.25</td>
</tr>
<tr>
<td>9.2</td>
<td>3.83</td>
<td>0.12</td>
</tr>
<tr>
<td>16.9</td>
<td>3.47</td>
<td>0.21</td>
</tr>
<tr>
<td>24.3</td>
<td>3.77</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Values represent the mean and standard deviation of triplicates.

Example 6
Homogeneous N-Terminal Amino Acid Sequence of EN-apyrase

Among the top 5 clones clone 350 was chosen to produce EN-apyrase from the CHO cell line. Conditioned medium was harvested from the sCHO-S/sC-APT-R 2X cell culture of clone 350 harboring pCS-APT-WPRE (new ori) to purify EN-apyrase. Purification was performed in the same way using DEAE and Heparin chromatographies as in Preparation A. Purified EN-apyrase was subjected to 4-12% SDS-PAGE gel in the presence of 2-mercaptoethanol and gave a single band at 70 kDa associated with apyrase activity. After completion of electrophoresis, the gel was immersed into a transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 0.05 mM chlorobutanol, 10% methanol, pH 7.2) for 5 minutes, overlaid to PVDF membrane (Immovilon, Millipore), which has previously been immersed successively into 100% methanol and the transfer buffer, and the protein was transferred with XCell II™ Blot Module (Invitrogen) at 160 mA for 1 hour. The PVDF membrane after transfer was washed with water, stained with Coomassie® Brilliant Blue R-250 Staining Solution (Bio-Rad) for 1 minute and washed with distilled water.

The stained band at 70 kDa was excised and the membrane segment was analyzed by Edman Sequence Analysis. The N-terminal amino acid sequence was a single species and its N-amino acid residues were determined as: Glu-Val-Leu-Pro-Pro-Gly-Leu-Lys-Tyr-Gly-Ile; thus cleavage occurs at position 28 SEQ ID NO:9. This represented only 40% of the product in HEK293T cells producing HEK-sol-CD39L3-01.

Example 7
Carbohydrate Analysis of EN-apyrase

Carbohydrates from EN-apyrase vs. sol CD39L3 (HEK-sol-CD39L3-01) were analyzed by isoelectric focusing. EN-apyrases show substantially more glycosylation and with less heterogeneity as demonstrated by a pi in a range of about 3.0 to about 4.5 vs. a range of about 5.0 to about 6.0 for CD39L3-01. See Figures 10A and B. The higher molecular weight due to enhanced glycosylation was confirmed by SDS-PAGE.
Example 8

Improved Purification Protocol

[0092] An improved purification protocol was developed based upon new properties of the EN-apyrases. A two-ion exchange chromatography (ANX and SP) protocol was used instead of an ion exchange (DEAE) and an affinity chromatography (Heparin) as used in Preparation A and Example 6.

[0093] CHO cells transformed with an EN-apyrase construct comprising SEQ ID NO: 10 were cultured generally as described above and the media was harvested through a 1.4 ft² 60M02 Depth Filter (Cuno, CT). The filter was washed prior to use with WFI water and was blown down with compressed air to maximize volumetric recovery. Clarified media was then filtered through a 0.2 μm filter and collected in a sterile bag.

[0094] For viral inactivation, the load (1.6 L clarified media, V17) was diluted with an equal volume (1.6 L) of WFI water. A solution of Triton® X-100 (320 mL of 11%) was added (1% final) and the resulting solution was incubated at ambient temperature for 30 minutes.

[0095] Anion Exchange Chromatography. The viral inactivated culture media (3.52 L) was applied at a flow rate of 13 ml/min to 80 mL ANX Sepharose FF (GE Healthcare) column equilibrated with 10 mM Tris-HCl, pH 7.4. The load was applied to the column and the flow-thru, plus wash, was collected (3.7 L). A second wash of 10 mM Tris, 140 mM NaCl, pH 7.4 was performed and collected (580 mL). The protein was eluted with 10 mM Tris, 230 mM NaCl, pH 7.4 and collected (500 mL). Finally, the column was stripped of remaining protein with 1 M NaCl and this too, was collected (450 mL).

[0096] Buffer Exchange and Cation Exchange Chromatography. The collected ANX 140 mM - 230 mM elution volume was buffer exchanged (-10 volumes) in continuous mode using a Pellicon Biomax 30 50 cm² into 20 mM citrate, pH 4.80. The buffer exchanged load (1.0 L) was applied to 80 mL SP-Sepharose FF (GE Healthcare) column equilibrated in 20 mM citrate, pH 4.80 and the flow-thru, plus wash, collected (1.2 L). A wash step was performed with 20 mM citrate, pH 5.10, collected (220 mL). The column was stripped of remaining protein with 20 mM citrate, pH 6.0 and this too, was collected (200 mL).

[0097] Yield and Purity Analysis. EN-apyrase yield was calculated to be greater than 80% by UV/vis absorption and by ELISA as presented.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>[Protein] (mg/mL)</th>
<th>Total Protein (mg)</th>
<th>Step Yield (%)</th>
<th>Overall Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified media</td>
<td>1600</td>
<td>*0.0458</td>
<td>*73.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ANX 140 wash</td>
<td>580</td>
<td>0.26</td>
<td>150.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANX 230 elute pool</td>
<td>500</td>
<td>0.32</td>
<td>160</td>
<td>218</td>
<td>218</td>
</tr>
<tr>
<td>BE ANX elute/SP load</td>
<td>1000</td>
<td>0.10</td>
<td>100</td>
<td>62.5</td>
<td>136</td>
</tr>
<tr>
<td>SP pH 4.8 flow-thru</td>
<td>1200</td>
<td>0.05</td>
<td>60</td>
<td>60.0</td>
<td>81.9</td>
</tr>
<tr>
<td>SP pH 5.1 wash</td>
<td>220</td>
<td>0.01</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP pH 6.0 strip</td>
<td>200</td>
<td>0.10</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[0098] SDS-PAGE analysis on each purification step was performed (Figure 11). Purity was analyzed in SD-PAGE gel after 20x concentration using YM-30 2 ml centrifuge concentrators.

Example 9

Purification of EN-apyrase with Improved Recovery of Heterogeneously Glycosylated EN-apyrase

[0099] The media from cells cultured as described in Example 2 was harvested through a 1.4 ft² 60M02 Depth Filter (Cuno, CT). The filter was washed prior to use with WFI water and was blown down with compressed air to maximize volumetric recovery. Clarified media was then filtered through a 0.2 µm filter and collected in a sterile bag.

[0100] For viral inactivation, the load (100 mL clarified media, VI9) was diluted with an equal volume (100 mL) of Milli-Q water. A solution of Triton® X-100 (20mL of 11%) was added (1% final) and the resulting solution was incubated at ambient temperature for 30 minutes.

[0101] Anion Exchange Chromatography. Viral inactivated culture media (220 mL) of EN-apyrase was applied at a flow rate of 5 ml/min to 5 mL ANX Sepharose FF (GE Healthcare) column equilibrated with 10 mM Tris-HCl, pH 7.4. The load was applied to the column and the flow-thru, plus wash, was collected (260 mL). The protein was eluted with 10 mM Tris, 230 mM NaCl, pH 7.4 and collected (50 mL). By skipping the wash step and direct elution with 230 mM NaCl there was no significant loss of apyrase from the anion exchange chromatography. Finally, the column was stripped of remaining protein with 1 M NaCl and this too, was collected (30 mL). Western blot analysis on 1 M NaCl strip showed almost no detection of apyrase in the fraction.
Buffer Exchange and Cation Exchange Chromatography. The collected ANX 230 mM elution volume was buffer exchanged (~3 volumes) through 1 L G25 column into 20 mM citrate, pH 4.6. The buffer exchanged load (150 mL) was applied to 5 mL SP-Sepharose FF (GE Healthcare) column equilibrated in 20 mM citrate, pH 4.6 and the flow-thru, plus wash, collected (170 mL). A wash step was performed with 20 mM citrate, pH 4.8, collected (40 mL). It was additional achievement that lower molecular weight impurities were removed by lowering flow-thru pH from 4.8 to 4.6. Although a portion of apyrase was in pH 4.8 elute the total amount was less than 10% of pH 4.6 flow-thru. Another wash step was performed with 20 mM citrate, pH 5.1, collected (40 mL). The column was stripped of remaining protein with 20 mM citrate, pH 6.0 and this too, was collected (40 mL).

Its purity was analyzed on 4-12% SDS-PAGE gel after 20x concentration using Omega 3 kDa 2 ml centrifuge concentrators.

With this purification scheme almost all of heterogeneously glycosylated EN-apyrase was collected from ANX chromatography by omitting 120 mM and/or 140 mM NaCl wash step. Also by reducing 0.2 pH unit on SP chromatography achieved additionally was a higher purity of apyrase (>95%). From this two step purification the overall recovery of apyrase was greater than 90%.

Example 10

EN-apyrase shows prolonged half life and improved Pharmacodynamics

5CHO-S/sC-APT-R 2X cells were cultured at pH 7.4, 34°C in the presence of glucose at 4 g/L and glutamine at 2 mM. The EN-apyrase was purified via the 2-step ion exchange process of Example 9 and demonstrated a higher average molecular weight vs. HEK-sol-CD39L3-01, resulting from more abundant glycosylation.

As a control, pharmacokinetic studies were conducted in rats where single bolus sol-CD39L3 expressed in HEK cells, was intravenously injected (0.75 mg/kg, n=3 per time point). Serum samples were examined for ADPase and ATPase activity. The experimental data best fit biphasic exponential curves for either enzyme activity. The distribution phase half-life (T1/2) of this apyrase was calculated to be 40 min (Figure 12). Approximately 50% of apyrase activity was cleared from the circulation during this phase. This apyrase has a plasma elimination T1/2 of 20 h. In contrast, EN-apyrase (administered at 0.25 mg/kg) retained >50% of the initial activity at 24h, increasing the effective in vivo activity by >10x as shown in Figure 13.

The levels of active apyrase as measured by the effect of the EN-apyrase vs. HEK-sol-CDL3-sol on ADP-induced platelet aggregation in platelet rich plasma were determined at
various time points after single bolus administration to rabbits (0.75 and 0.25 mg/kg of HEK-sol-CD39L3-01 and EN-apyrase respectively). To estimate inhibition of platelet aggregation by the apyrases more accurately, blood samples were heparinized, rather than citrated, in order to maintain physiological calcium concentrations. The data demonstrate that while soluble CD39L3 (e.g., HEK-sol-CD39L3-01) retained only 50% inhibition of ADP-induced platelet aggregation at 6h, a 3x lower concentration (0.25 vs. 0.75 mg/kg) of EN-apyrase retained 90% inhibition of ADP-induced platelet aggregation at 6h as shown in Figure 14.

[0108] Similar improvement of pharmacokinetics by EN-apyrase was observed in pigs as shown in Figures 15 and 16. The >10 fold improvement in the pharmacokinetics of EN-apyrase would likely reduce the effective dose.
Claims

1. An EN-apyrase, wherein the EN-apyrase is a soluble CD39L3 or a homolog thereof, has a homogeneous N-terminus and has an average isoelectric point in the range of about 3.0 to about 4.5; and/or wherein said EN-apyrase has an \textit{in vivo} half life in rabbits or pigs at least twice that of HEK sol-CDE39L3-01, measured by apyrase assay.

2. The EN-apyrase of claim 1 which is produced by a transformed CHO cell line.

3. The EN-apyrase of claim 1 or 2 wherein N terminus has the sequence EVLP.

4. The EN-apyrase of claim 3 which comprises SEQ ID NO:5 beginning at position 7 thereof or is a homolog thereof.

5. A nucleic acid construct comprising a nucleotide sequence encoding a signal sequence, a linker, and a soluble apyrase, wherein the linker has the sequence EVLP as its C-terminus and wherein said linker or a portion thereof may represent a sequence present in the native soluble apyrase.

6. The construct of claim 5, further comprising an operably linked promoter functional in CHO cells.

7. The construct of claim 5 or 6 wherein the soluble apyrase is a soluble CD39L3 or homolog thereof.

8. The construct of claim 7 wherein the soluble apyrase is of SEQ ID NO:5, starting at position 7 thereof.

9. CHO cells modified to contain the construct of any of claims 6-8.

10. A method to prepare an EN-apyrase which method comprises culturing the cells of claim 9, and collecting the cultured medium.
11. The method of claim 10 wherein during culturing the medium maintains a glutamine concentration at about 1.5-4 mM and a pH of between 7.0 and 7.8 and wherein the temperature is shifted from 37°C to 31°C-35°C at days 4-6 of culturing.

12. The method of claim 11, wherein during culturing the medium maintains a glutamine concentration at about 2 mM and a pH of 7.4 and wherein the temperature of the culture is shifted from 37°C to 34°C at day 5 of culturing.


14. A pharmaceutical composition comprising the EN-apyrase of claim 1.

15. A pharmaceutical composition comprising the EN-apyrase of claim 13.

16. A method to treat conditions benefited by apyrase activity, which method comprises administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim 14 or 15.

17. A method to purify the EN-apyrase of any of claims 1-4 or 13 which method comprises subjecting culture medium containing said EN-apyrase to anion exchange chromatography, followed by treating EN-apyrase containing eluate from said anion exchange chromatography to purification by cation exchange chromatography.
Figure 3

Figure 4

3 / 13
Figure 5

Figure 6
Figure 9

Stability Study sCHO-S/sC-APT-R Clone # 350

Ex-aryrase Production (mg/L)

Generation
Figure 11
Figure 12
Figure 13
Figure 16
INTERNATIONAL SEARCH REPORT

INTERNATIONAL CLASSIFICATION OF SUBJECT MATTER

IPCB - C07K 14/435 (2011.01)
USPC - 530/350

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)
USPC 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 424/94.6; 435/69.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST=DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES, OP=ADD; Google Scholar; CD39L3 isoelectric point, CD39L3 pi, Ectonucleoside triphosphate diphosphohydrolase 3 isolectric point, Ectonucleoside triphosphate diphosphohydrolase 3 pi, NTPDase 3 isolectric point, NTPDase3 isolectric point, NTPDase3 pi)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>Y</td>
<td>US 7,390,485 B2 (JEONG et al.), 24 June 2008 (24.06.2008); col 2, ln 33-45; col 15, ln 35-43; col 18, ln 46-67; col 27, ln 31-49; Table 3; SEQ ID NO: 71, 72, 76</td>
<td>1-8, 14</td>
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<td>Y</td>
<td>US 5,322,678 A (MORGAN et al.), 21 June 1994 (21.06.1994); col 7, ln 33-67; col 8, ln 1-26</td>
<td>1-4, 14</td>
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Date of the actual completion of the international search
21 April 2011 (21.04.2011)

Date of mailing of the international search report
06 MAY 2011

Form PCT/ISA/210 (second sheet) (July 2009)
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<td>This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:</td>
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<td>1.</td>
<td>□ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:</td>
</tr>
<tr>
<td></td>
<td>□ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:</td>
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<tr>
<td>3.</td>
<td>□ Claims Nos.: 9-13 and 15-17 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).</td>
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<td>This International Searching Authority found multiple inventions in this international application, as follows:</td>
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<td>1.</td>
<td>□ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.</td>
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<td>2.</td>
<td>□ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.</td>
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<td>3.</td>
<td>□ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:</td>
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<tr>
<td>4.</td>
<td>□ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:</td>
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</table>

**Remark on Protest**

- □ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- □ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- □ No protest accompanied the payment of additional search fees.