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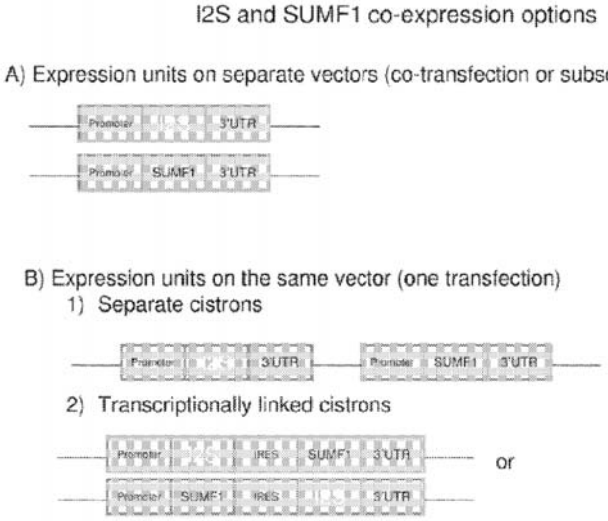
(54) Title: METHOD OF PRODUCING RECOMBINANT IDURONATE-2-SULFATASE



(57) Abstract: The present invention provides, among other things, methods and compositions for large-scale production of recombinant I2S protein using suspension culture of mammalian cells in serum-free medium. In particular, the present invention uses mammalian cells co-express a recombinant I2S protein and a formylglycine generating enzyme (FGE).

METHOD OF PRODUCING RECOMBINANT IDURONATE-2-SULFATASE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 61/666,712, filed June 29, 2012; the entirety of which is hereby incorporated by reference.

SEQUENCE LISTING

[0002] The present specification makes reference to a Sequence Listing submitted in electronic form as an ASCII .txt file named "2006685-0339_SEQ_LIST" on June 27, 2013. The .txt file was generated on June 25, 2013 and is 21 KB in size. The entire contents of the Sequence Listing are herein incorporated by reference.

BACKGROUND

[0003] Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is an X-chromosome-linked recessive lysosomal storage disorder that results from a deficiency in the enzyme iduronate-2-sulfatase (I2S). I2S cleaves the terminal 2-O-sulfate moieties from the glycosaminoglycans (GAG) dermatan sulfate and heparan sulfate. Due to the missing or defective I2S enzyme in patients with Hunter syndrome, GAG progressively accumulate in the lysosomes of a variety of cell types, leading to cellular engorgement, organomegaly, tissue destruction, and organ system dysfunction.

[0004] Generally, physical manifestations for people with Hunter syndrome include both somatic and neuronal symptoms. For example, in some cases of Hunter syndrome, central nervous system involvement leads to developmental delays and nervous system problems. While the non-neuronal symptoms of Hunter Syndrome are generally absent at birth, over time the progressive accumulation of GAG in the cells of the body can have a dramatic impact on the peripheral tissues of the body. GAG accumulation in the peripheral tissue leads to a distinctive coarseness in the facial features of a patient and is responsible for the prominent forehead, flattened bridge and enlarged tongue, the defining hallmarks of a Hunter patient. Similarly, the accumulation of GAG can adversely affect the organ systems of the body. Manifesting initially as a thickening of the wall of the heart, lungs and airways,

and abnormal enlargement of the liver, spleen and kidneys, these profound changes can ultimately lead to widespread catastrophic organ failure. As a result, Hunter syndrome is always severe, progressive, and life-limiting.

[0005] Enzyme replacement therapy (ERT) is an approved therapy for treating Hunter syndrome (MPS II), which involves administering exogenous replacement I2S enzyme to patients with Hunter syndrome.

SUMMARY OF THE INVENTION

[0006] The present invention provides, among other things, an improved method for large scale production of recombinant I2S enzyme to facilitate effective treatment of Hunter syndrome. Prior to the present invention, roller bottle adherent culture system using serum-containing medium has been successfully developed to produce recombinant I2S at large scale. The inventors of the present application however developed a system that can effectively cultivate mammalian cells co-expressing I2S and formylglycine generating enzyme (FGE) in suspension in a large scale vessel using animal-component free, chemically-defined medium to efficiently produce a large quantity of recombinant I2S enzyme. Unexpectedly, a recombinant I2S enzyme produced using the animal-free suspension culturing system also has significantly improved enzymatic activity because the recombinant I2S produced in this fashion has an unusually high level of C_{α}-formylglycine (FGly) (e.g., above 70% and up to 100%), which is required for the activity of I2S. In addition, the recombinant I2S enzyme produced according to the present invention has distinct characteristics such as sialic acid content and glycan map, which may improve bioavailability of the recombinant I2S protein. Moreover, the animal free culture system simplifies the downstream purification process and reduces or eliminates serum-originated contaminants such as fetuin. Thus, the present invention provides a large scale production system that is more efficient, cost-effective, reproducible, safer and produces more potent recombinant I2S.

[0007] Thus, in one aspect, the present invention provides a method for large-scale production of recombinant iduronate-2-sulfatase (I2S) protein in mammalian cells by culturing mammalian cells co-expressing a recombinant I2S protein and a formylglycine generating enzyme (FGE) in suspension in a large-scale culture vessel containing medium lacking serum. In some embodiments, the culturing step involves a perfusion process.

[0008] In another aspect, the present invention provides a method for large-scale production of recombinant iduronate-2-sulfatase (I2S) protein in mammalian cells, comprising culturing mammalian cells co-expressing a recombinant I2S protein and a formylglycine generating enzyme (FGE) in a large-scale culture vessel containing medium lacking serum under conditions such that the cells, on average, produce the recombinant I2S protein at a specific productivity rate of great than about 15 picogram/cell/day and further wherein the produced recombinant I2S protein, on average, comprises at least about 60% conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_{α}-formylglycine. In some embodiments, the culturing step involves a perfusion process.

[0009] In some embodiments, the perfusion process has a perfusion rate ranging from about 0.5-2 volume of fresh medium/working volume of reactor/day (VVD) (e.g., about 0.5-1.5 VVD, about 0.75-1.5 VVD, about 0.75-1.25 VVD, about 1.0-2.0 VVD, about 1.0-1.9 VVD, about 1.0-1.8 VVD, about 1.0-1.7 VVD, about 1.0-1.6 VVD, about 1.0-1.5 VVD, about 1.0-1.4 VVD, about 1.0-1.3 VVD, about 1.0-1.2 VVD, about 1.0-1.1 VVD). In some embodiments, the perfusion process has a perfusion rate of about 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.05, 1.10, 1.15, 1.2, 1.25, 1.3, 1.35, 1.4, 1.45, 1.5, 1.55, 1.6, 1.65, 1.7, 1.75, 1.8, 1.85, 1.9, 1.95, or 2.0 VVD.

[0010] In some embodiments, the perfusion process has a cell specific perfusion rate ranging from about 0.05-5 nanoliter per cell per day (nL/cell/day) (e.g., about 0.05-4 nL/cell/day, about 0.05-3 nL/cell/day, about 0.05-2 nL/cell/day, about 0.05-1 nL/cell/day, about 0.1-5 nL/cell/day, about 0.1-4 nL/cell/day, about 0.1-3 nL/cell/day, about 0.1-2 nL/cell/day, about 0.1-1 nL/cell/day, about 0.15-5 nL/cell/day, about 0.15-4 nL/cell/day, about 0.15-3 nL/cell/day, about 0.15-2 nL/cell/day, about 0.15-1 nL/cell/day, about 0.2-5 nL/cell/day, about 0.2-4 nL/cell/day, about 0.2-3 nL/cell/day, about 0.2-2 nL/cell/day, about 0.2-1 nL/cell/day, about 0.25-5 nL/cell/day, about 0.25-4 nL/cell/day, about 0.25-3 nL/cell/day, about 0.25-2 nL/cell/day, about 0.25-1 nL/cell/day, about 0.3-5 nL/cell/day, about 0.3-4 nL/cell/day, about 0.3-3 nL/cell/day, about 0.3-2 nL/cell/day, about 0.3-1 nL/cell/day, about 0.35-5 nL/cell/day, about 0.35-4 nL/cell/day, about 0.35-3 nL/cell/day, about 0.35-2 nL/cell/day, about 0.35-1 nL/cell/day, about 0.4-5 nL/cell/day, about 0.4-4 nL/cell/day, about 0.4-3 nL/cell/day, about 0.4-2 nL/cell/day, about 0.4-1 nL/cell/day, about 0.45-5 nL/cell/day, about 0.45-4 nL/cell/day, about 0.45-3 nL/cell/day, about 0.45-2 nL/cell/day, about 0.45-1 nL/cell/day, about 0.5-5 nL/cell/day, about 0.5-4 nL/cell/day, about

0.5-3 nL/cell/day, about 0.5-2 nL/cell/day, about 0.5-1 nL/cell/day). In some embodiments, the perfusion process has a cell specific perfusion rate of about 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0 nL/cell/day.

[0011] In some embodiments, the cells cultivated according to the present invention, on average, produce the recombinant I2S protein at a specific productivity rate of great than about 20 picogram/cell/day (e.g., greater than about 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 picogram/cell/day). In some embodiments, the cells cultivated according to the present invention produce the recombinant I2S protein at an average harvest titer of at least 6 mg per liter per day (mg/L/day) (e.g., at least 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, or 500 mg/L/day, or more).

[0012] In some embodiments, the produced recombinant I2S protein according to a method of the invention comprises at least about 70% (e.g., at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%) conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_{α}-formylglycine (FGly).

[0013] In some embodiments, mammalian cells suitable for the present invention are human cells. In some embodiments, mammalian cells suitable for the present invention are CHO cells.

[0014] In some embodiments, a large-scale culture vessel suitable for the present invention is a bioreactor. In some embodiments, a suitable bioreactor is at a scale of or greater than 10L, 200L, 500L, 1000L, 1500L, 2000L, 2500L, 3000L.

[0015] In some embodiments, a medium suitable for the present invention lacks animal-derived components. In some embodiments, a suitable medium is chemically-defined medium. In some embodiments, a suitable medium is protein free.

[0016] In some embodiments, a medium suitable for the present invention contains at least one redox-modulator. In some embodiments, a redox-modulator suitable for the present invention is selected from the group consisting of glutathione, glucose-6-phosphate, carnosine, carnosol, sulforaphane, tocopherol, ascorbate, dehydroascorbate, selenium, 2-

mercaptoenthanol, N-acetylcysteine, cysteine, riboflavin, niacin, folate, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADP), and combination thereof. In some embodiments, a suitable redox-modulator is cysteine. In some embodiments, the cysteine is at a concentration ranging from about 0.1 mg/L to about 65 mg/L (e.g., 1-50 mg/L, 1-40 mg/L, 1-30 mg/l, 1-20 mg/L, 1-10 mg/L). In some embodiments, a suitable redox-modulator is 2-mercaptoenthanol. In some embodiments, the 2-mercaptoenthanol is at a concentration ranging from about 0.001 mM to about 0.01 mM (e.g., about 0.001-0.008 mM, about 0.001-0.007 mM, about 0.001-0.006 mM, about 0.001-0.005 mM, about 0.001-0.004 mM, about 0.001-0.003 mM, about 0.001-0.002 mM). In some embodiments, a suitable redox-modulator is N-acetylcysteine. In some embodiments, the N-acetylcysteine is at a concentration ranging from about 3 mM to about 9 mM (e.g., about 3-8 mM, about 3-7 mM, about 3-6 mM, about 3-5 mM, about 3-4 mM).

[0017] In some embodiments, a medium suitable for the present invention contains at least one growth-modulator. In some embodiments, a suitable growth-modulator is hypoxanthine. In some embodiments, the hypoxanthine is at a concentration ranging from about 0.1 mM to about 10 mM (e.g., about 0.1-9 mM, about 0.1-8 mM, about 0.1-7 mM, about 0.1-6 mM, about 0.1-5 mM, about 0.1-4 mM, about 0.1-3 mM, about 0.1-2 mM, about 0.1-1 mM). In some embodiments, a suitable growth-modulator is thymidine. In some embodiments, the thymidine is at a concentration ranging from about 1 mM to about 100 mM (e.g., about 1-90 mM, about 1-80 mM, about 1-70 mM, about 1-60 mM, about 1-50 mM, about 1-40 mM, about 1-30 mM, about 1-20 mM, about 1-10 mM).

[0018] In some embodiments, the medium has a pH ranging from about 6.8 – 7.5 (e.g., about 6.9-7.4, about 6.9-7.3, about 6.95-7.3, about 6.95-7.25, about 7.0-7.3, about 7.0-7.25, about 7.0-7.2, about 7.0-7.15, about 7.05-7.3, about 7.05-7.25, about 7.05-7.15, about 7.05-7.20, about 7.10-7.3, about 7.10-7.25, about 7.10-7.20, about 7.10-7.15). In some embodiments, the medium has a pH of about 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, 7.4, 7.45, or 7.5.

[0019] In some embodiments, the culturing step of various methods described herein include a growth phase and a production phase. In some embodiments, the mammalian cells are cultured at a temperature ranging from about 30-37 °C (e.g., about 31-37 °C, about 32-37 °C, about 33-37 °C, about 34-37 °C, about 35-37 °C, about 36-37 °C). In some embodiments, the mammalian cells are cultured at a temperature of approximately 30 °C, 31 °C, 32 °C, 33

°C, 34 °C, 35 °C, 36 °C, or 37 °C. Any of the temperatures described herein may be used for growth and/or production phase. In some embodiments, the mammalian cells are cultured at different temperatures during the growth phase and the production phase. In some embodiments, the mammalian cells are cultured at substantially the same temperatures during the growth phase and the production phase. Any of the medium pH described herein may be used for growth and/or production phase. In some embodiments, the medium pH for the growth phase and the production phase is different. In some embodiments, the medium pH for the growth phase and the production phase is substantially the same.

[0020] In some embodiments, the mammalian cells are maintained at a viable cell density ranging from about 1.0-50 X 10^6 viable cells/mL during the production phase (e.g., about 1.0-40 X 10^6 viable cells/mL, about 1.0-30 X 10^6 viable cells/mL, about 1.0-20 X 10^6 viable cells/mL, about 1.0-10 X 10^6 viable cells/mL, about 1.0-5 X 10^6 viable cells/mL, about 1.0-4.5 X 10^6 viable cells/mL, about 1.0-4 X 10^6 viable cells/mL, about 1.0-3.5 X 10^6 viable cells/mL, about 1.0-3 X 10^6 viable cells/mL, about 1.0-2.5 X 10^6 viable cells/mL, about 1.0-2.0 X 10^6 viable cells/mL, about 1.0-1.5 X 10^6 viable cells/mL, about 1.5-10 X 10^6 viable cells/mL, about 1.5-5 X 10^6 viable cells/mL, about 1.5-4.5 X 10^6 viable cells/mL, about 1.5-4 X 10^6 viable cells/mL, about 1.5-3.5 X 10^6 viable cells/mL, about 1.5-3.0 X 10^6 viable cells/mL, about 1.5-2.5 X 10^6 viable cells/mL, about 1.5-2.0 X 10^6 viable cells/mL).

[0021] In some embodiments, the production phase is lasted for about 5-90 days (e.g., about 5-80 days, about 5-70 days, about 5-60 days, about 5-50 days, about 5-40, about 5-30 days, about 5-20 days, about 5-15 days, about 5-10 days, about 10-90 days, about 10-80 days, about 10-70 days, about 10-60 days, about 10-50 days, about 10-40 days, about 10-30 days, about 10-20 days, about 15-90 days, about 15-80 days, about 15-70 days, about 15-60 days, about 15-50 days, about 15-40 days, about 15-30 days). In some embodiments, the production phase is lasted for about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 days.

[0022] In various embodiments, mammalian cells express a recombinant I2S protein having an amino acid sequence at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) identical to SEQ ID NO:1. In some embodiments, an inventive method described herein is used to produce a recombinant I2S protein having an amino acid sequence identical to SEQ ID NO:1.

[0023] In various embodiments, mammalian cells express an FGE protein having an amino acid sequence at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) identical to SEQ ID NO:5. In some embodiments, a mammalian cell expresses an FGE protein having an amino acid sequence identical to SEQ ID NO:5.

[0024] In various embodiments, mammalian cells contain one or more exogenous nucleic acids encoding the recombinant I2S protein and/or the FGE. In some embodiments, the one or more exogenous nucleic acids are integrated in the genome of the cells. In some embodiments, the one or more exogenous nucleic acids are present on one or more extra-chromosomal constructs. In some embodiments, mammalian cells used in a method of the present invention over-express the recombinant I2S protein. In some embodiments, mammalian cells used in a method of the present invention over-express the FGE.

[0025] In various embodiments, an inventive method according to the present invention further includes a step of harvesting the recombinant I2S protein.

[0026] In yet another aspect, the present invention provides a recombinant iduronate-2-sulfatase (I2S) protein produced using a method described herein. In some embodiments, the present invention provides a preparation of recombinant I2S protein, in which the recombinant I2S protein has at least about 70% (e.g., at least about 77%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) conversion of the cysteine residue corresponding to Cys59 of human I2S (SEQ ID NO:1) to C_α-formylglycine (FGly). In some embodiments, the present invention provides a preparation of recombinant I2S protein, in which the recombinant I2S protein has substantially 100% conversion of the cysteine residue corresponding to Cys59 of human I2S (SEQ ID NO:1) to C_α-formylglycine (FGly). In some embodiments, the present invention provides a preparation of recombinant iduronate-2-sulfatase (I2S) protein, said recombinant I2S protein having an amino acid sequence at least about 50% (e.g., at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%) identical to SEQ ID NO:1. In some embodiments, the recombinant I2S protein has an amino acid sequence identical to SEQ ID NO:1.

[0027] In some embodiments, the recombinant I2S protein has specific activity of at least about 20 U/mg, 30 U/mg, 40 U/mg, 50 U/mg, 60 U/mg, 70 U/mg, 80 U/mg, 90 U/mg, or

100 U/mg mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

[0028] Among other things, the present invention also provides a pharmaceutical composition containing a recombinant I2S protein described in various embodiments herein and a pharmaceutically acceptable carrier and a method of treating Hunter syndrome by administering into a subject in need of treatment recombinant I2S protein described herein or a pharmaceutical composition containing the same.

[0029] As used herein, the terms "I2S protein," "I2S," "I2S enzyme," or grammatical equivalents, refer to a preparation of recombinant I2S protein molecules unless otherwise specifically indicated.

[0030] As used in this application, the terms "about" and "approximately" are used as equivalents. Any numerals used in this application with or without about/approximately are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art.

[0031] Other features, objects, and advantages of the present invention are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating embodiments of the present invention, is given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] The Figures described below, that together make up the Drawing, are for illustration purposes only, not for limitation.

[0033] *Figure 1* depicts the amino acid sequence (SEQ ID NO: 1) encoding the mature form of human iduronate-2-sulfatase (I2S) protein and indicates potential sites within the protein sequence for N-linked glycosylation and cysteine conversion.

[0034] *Figure 2* depicts exemplary construct designs for co-expression of I2S and FGE (i.e., SUMF1). (A) Expression units on separate vectors (for co-transfection or

subsequent transfections); (B) Expression units on the same vector (one transfection): (1) Separate cistrons and (2) Transcriptionally linked cistrons.

[0035] *Figure 3* demonstrates exemplary expression of full length recombinant I2S by SDS-PAGE generated using cell lines grown under either serum-free or serum based cell culture conditions, as compared to an I2S reference standard.

[0036] *Figure 4* shows an exemplary peptide map for a recombinant I2S enzyme produced from the I2S-AF 2D cell line grown under serum-free culture conditions (top panel), versus a reference recombinant I2S enzyme

[0037] *Figure 5* depicts an exemplary glycan profile generated for recombinant I2S enzyme produced using the I2S-AF 2D and 4D cell lines grown under serum-free cell culture conditions as compared to a reference recombinant I2S enzyme.

[0038] *Figure 6* depicts an exemplary charge profile generated for recombinant I2S enzyme produced using the I2S-AF 2D cell line grown under serum-free cell culture conditions as compared to a reference recombinant I2S enzyme.

DEFINITIONS

[0039] In order for the present invention to be more readily understood, certain terms are first defined. Additional definitions for the following terms and other terms are set forth throughout the specification.

[0040] *Amino acid*: As used herein, term "amino acid," in its broadest sense, refers to any compound and/or substance that can be incorporated into a polypeptide chain. In some embodiments, an amino acid has the general structure H_2N–C(H)(R)–COOH. In some embodiments, an amino acid is a naturally occurring amino acid. In some embodiments, an amino acid is a synthetic amino acid; in some embodiments, an amino acid is a D-amino acid; in some embodiments, an amino acid is an L-amino acid. "Standard amino acid" refers to any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "Nonstandard amino acid" refers to any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or obtained from a natural source. As used herein, "synthetic amino acid" encompasses chemically modified amino acids, including but

not limited to salts, amino acid derivatives (such as amides), and/or substitutions. Amino acids, including carboxy- and/or amino-terminal amino acids in peptides, can be modified by methylation, amidation, acetylation, protecting groups, and/or substitution with other chemical groups that can change the peptide's circulating half-life without adversely affecting their activity. Amino acids may participate in a disulfide bond. Amino acids may comprise one or posttranslational modifications, such as association with one or more chemical entities (*e.g.*, methyl groups, acetate groups, acetyl groups, phosphate groups, formyl moieties, isoprenoid groups, sulfate groups, polyethylene glycol moieties, lipid moieties, carbohydrate moieties, biotin moieties, *etc.* In some embodiments, amino acids of the present invention may be provided in or used to supplement medium for cell cultures. In some embodiments, amino acids provided in or used to supplement cell culture medium may be provided as salts or in hydrate form.

[0041] *Approximately:* As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0042] *Batch culture*: The term "batch culture" as used herein refers to a method of culturing cells in which all the components that will ultimately be used in culturing the cells, including the medium (see definition of "medium" below) as well as the cells themselves, are provided at the beginning of the culturing process. Thus, a batch culture typically refers to a culture allowed to progress from inoculation to conclusion without refeeding the cultured cells with fresh medium. A batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and optionally purified.

[0043] *Bioavailability:* As used herein, the term "bioavailability" generally refers to the percentage of the administered dose that reaches the blood stream of a subject.

[0044] *Biologically active*: As used herein, the phrase "biologically active" refers to a characteristic of any substance that has activity in a biological system (*e.g.*, cell culture, organism, *etc.*). For instance, a substance that, when administered to an organism, has a

biological effect on that organism, is considered to be biologically active. Biological activity can also be determined by *in vitro* assays (for example, in vitro enzymatic assays such as sulfate release assays). In particular embodiments, where a protein or polypeptide is biologically active, a portion of that protein or polypeptide that shares at least one biological activity of the protein or polypeptide is typically referred to as a "biologically active" portion. In some embodiments, a protein is produced and/or purified from a cell culture system, which displays biologically activity when administered to a subject. In some embodiments, a protein requires further processing in order to become biologically active. In some embodiments, a protein requires posttranslational modification such as, but is not limited to, glycosylation (e.g., sialyation), farnysylation, cleavage, folding, formylglycine conversion and combinations thereof, in order to become biologically active. In some embodiments, a protein produced as a proform (i.e. immature form), may require additional modification to become biologically active.

[0045] *Bioreactor*: The term "bioreactor" as used herein refers to a vessel used for the growth of a host cell culture. A bioreactor can be of any size so long as it is useful for the culturing of mammalian cells. Typically, a bioreactor will be at least 1 liter and may be 10, 100, 250, 500, 1000, 2500, 5000, 8000, 10,000, 12,0000 liters or more, or any volume in between. Internal conditions of a bioreactor, including, but not limited to pH, osmolarity, CO_2 saturation, O_2 saturation, temperature and combinations thereof, are typically controlled during the culturing period. A bioreactor can be composed of any material that suitable for holding cells in media under the culture conditions of the present invention, including glass, plastic or metal. In some embodiments, a bioreactor may be used for performing animal cell culture. In some embodiments, a bioreactor may be used for performing mammalian cell culture. In some embodiments, a bioreactor may used with cells and/or cell lines derived from such organisms as, but not limited to, mammalian cell, insect cells, bacterial cells, yeast cells and human cells. In some embodiments, a bioreactor is used for large-scale cell culture production and is typically at least 100 liters and may be 200, 500, 1000, 2500, 5000, 8000, 10,000, 12,0000 liters or more, or any volume in between. One of ordinary skill in the art will be aware of and will be able to choose suitable bioreactors for use in practicing the present invention.

[0046] *Cell density*: The term "cell density" as used herein refers to that number of cells present in a given volume of medium.

[0047] *Cell culture or culture*: These terms as used herein refer to a cell population that is gown in a medium under conditions suitable to survival and/or growth of the cell population. As will be clear to those of ordinary skill in the art, these terms as used herein may refer to the combination comprising the cell population and the medium in which the population is grown.

[0048] *Cultivation*: As used herein, the term "cultivation" or grammatical equvilents refers to a process of maintaining cells under conditions favoring growth or survival. The terms "cultivation" and "cell culture" or any synonyms are used inter-changeably in this application.

[0049] *Culture vessel*: As used herein, the term "culture vessel" refers to any container that can provide an aseptic environment for culturing cells. Exemplary culture vessels include, but are not limited to, glass, plastic, or metal containers.

[0050] *Dosage form:* As used herein, the terms "dosage form" and "unit dosage form" refer to a physically discrete unit of a therapeutic protein for the patient to be treated. Each unit contains a predetermined quantity of active material calculated to produce the desired therapeutic effect. It will be understood, however, that the total dosage of the composition will be decided by the attending physician within the scope of sound medical.

[0051] *Dosing regimen:* A "dosing regimen" (or "therapeutic regimen"), as that term is used herein, is a set of unit doses (typically more than one) that are administered individually to a subject, typically separated by periods of time. In some embodiments, a given therapeutic agent has a recommended dosing regiment, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which are separated from one another by a time period of the same length; in some embodiments, a dosing regime comprises a plurality of doses and at least two different time periods separating individual doses.

[0052] *Enzyme replacement therapy (ERT)*: As used herein, the term "enzyme replacement therapy (ERT)" refers to any therapeutic strategy that corrects an enzyme deficiency by providing the missing enzyme. In some embodiments, the missing enzyme is provided by intrathecal administration. In some embodiments, the missing enzyme is provided by infusing into bloodsteam. Once administered, enzyme is taken up by cells and transported to the lysosome, where the enzyme acts to eliminate material that has

accumulated in the lysosomes due to the enzyme deficiency. Typically, for lysosomal enzyme replacement therapy to be effective, the therapeutic enzyme is delivered to lysosomes in the appropriate cells in target tissues where the storage defect is manifest.

[0053] *Excipient*: As used herein , the term "excipient" referes to any inert substance added to a drug and/or formulation for the purposes of improving its physical qualities (i.e. consistency), pharmacokinetic properties (i.e. bioavailabity), pharmacodynamic properties and combinations thereof.

[0054] *Expression*: As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.*, by transcription); (2) processing of an RNA transcript (*e.g.*, by splicing, editing, 5' cap formation, and/or 3' end formation); (3) translation of an RNA into a polypeptide or protein; and/or (4) post-translational modification of a polypeptide or protein.

[0055] *Fed-batch culture*: The term "fed-batch culture" as used herein refers to a method of culturing cells in which additional components are provided to the culture at some time subsequent to the beginning of the culture process. The provided components typically comprise nutritional supplements for the cells which have been depleted during the culturing process. A fed-batch culture is typically stopped at some point and the cells and/or components in the medium are harvested and optionally purified.

[0056] *Fragment*: The term "fragment" as used herein refers to polypeptides and is defined as any discrete portion of a given polypeptide that is unique to or characteristic of that polypeptide. The term as used herein also refers to any discrete portion of a given polypeptide that retains at least a fraction of the activity of the full-length polypeptide. Preferably the fraction of activity retained is at least 10% of the activity of the full-length polypeptide. More preferably the fraction of activity retained is at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the activity of the full-length polypeptide. More preferably still the fraction of activity retained is at least 95%, 96%, 97%, 98% or 99% of the activity of the full-length polypeptide. Most preferably, the fraction of activity retained is 100% of the activity of the full-length polypeptide. The term as used herein also refers to any portion of a given polypeptide that includes at least an established sequence element found in the full-length polypeptide. Preferably, the sequence element spans at least 4-5, more preferably at

least about 10, 15, 20, 25, 30, 35, 40, 45, 50 or more amino acids of the full-length polypeptide.

[0057] *Gene*: The term "gene" as used herein refers to any nucleotide sequence, DNA or RNA, at least some portion of which encodes a discrete final product, typically, but not limited to, a polypeptide, which functions in some aspect of a cellular process. The term is not meant to refer only to the coding sequence that encodes the polypeptide or other discrete final product, but may also encompass regions preceding and following the coding sequence that modulate the basal level of expression, as well as intervening sequences ("introns") between individual coding segments ("exons"). In some embodiments, a gene may include regulatory sequences (*e.g.*, promoters, enhancers, polyadenylation sequences, termination sequences, Kozak sequences, TATA box, *etc.*) and/or modification sequences. In some embodiments, a gene may include references to nucleic acids that do not encode proteins but rather encode functional RNA molecules such as tRNAs, RNAi-inducing agents, *etc.*

[0058] *Gene product* or *expression product*: As used herein, the term "gene product" or "expression product" generally refers to an RNA transcribed from the gene (pre-and/or post-processing) or a polypeptide (pre- and/or post-modification) encoded by an RNA transcribed from the gene.

[0059] *Genetic control element*: The term "genetic control element" as used herein refers to any sequence element that modulates the expression of a gene to which it is operably linked. Genetic control elements may function by either increasing or decreasing the expression levels and may be located before, within or after the coding sequence. Genetic control elements may act at any stage of gene expression by regulating, for example, initiation, elongation or termination of transcription, mRNA splicing, mRNA editing, mRNA stability, mRNA localization within the cell, initiation, elongation or termination of translation, or any other stage of gene expression. Genetic control elements may function individually or in combination with one another.

[0060] *Homology*: As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%,

85%, 90%, 95%, or 99% identical. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% similar.

[0061] *Identity*: As used herein, the term "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two nucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or substantially 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4: 11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Various other sequence alignment programs are available and can be used to determine sequence identity such as, for example, Clustal.

[0062] *Improve, increase, or reduce*: As used herein, the terms "improve," "increase" or "reduce," or grammatical equivalents, indicate values that are relative to a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein. A "control individual" is an

individual afflicted with the same form of lysosomal storage disease as the individual being treated, who is about the same age as the individual being treated (to ensure that the stages of the disease in the treated individual and the control individual(s) are comparable).

[0063] *Integrated Viable Cell Density*: The term "integrated viable cell density" as used herein refers to the average density of viable cells over the course of the culture multiplied by the amount of time the culture has run. Assuming the amount of polypeptide and/or protein produced is proportional to the number of viable cells present over the course of the culture, integrated viable cell density is a useful tool for estimating the amount of polypeptide and/or protein produced over the course of the culture.

[0064] *Intrathecal administration*: As used herein, the term "intrathecal administration" or "intrathecal injection" refers to an injection into the spinal canal (intrathecal space surrounding the spinal cord). Various techniques may be used including, without limitation, lateral cerebroventricular injection through a burrhole or cisternal or lumbar puncture or the like. In some embodiments, "intrathecal administration" or "intrathecal delivery" according to the present invention refers to IT administration or delivery via the lumbar area or region, i.e., lumbar IT administration or delivery. As used herein, the term "lumbar region" or "lumbar area" refers to the area between the third and fourth lumbar (lower back) vertebrae and, more inclusively, the L2-S1 region of the spine.

[0065] *Isolated*: As used herein, the term "isolated" refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. As used herein, calculation of percent purity of isolated substances and/or entities should not include excipients (*e.g.*, buffer, solvent, water, *etc.*)

[0066] *Medium*: The terms as used herein refer to a solution containing nutrients which nourish growing cells. Typically, these solutions provide essential and non-essential amino acids, vitamins, energy sources, lipids, and trace elements required by the cell for minimal growth and/or survival. The solution may also contain components that enhance growth and/or survival above the minimal rate, including hormones and growth factors. In some embodiments, medium is formulated to a pH and salt concentration optimal for cell survival and proliferation. In some embodiments, medium may be a "chemically defined medium" – a serum-free media that contains no proteins, hydrolysates or components of unknown composition. In some embodiment, chemically defined medium is free of animal-derived components and all components within the medium have a known chemical structure. In some embodiments, medium may be a "serum based medium" – a medium that has been supplemented with animal derived components such as, but not limited to, fetal calf serum, horse serum, goat serum, donkey serum and/or combinations thereof.

[0067] *Metabolic waste product*: The term "metabolic waste product" as used herein refers to compounds produced by the cell culture as a result of normal or non-normal metabolic processes that are in some way detrimental to the cell culture, particularly in relation to the expression or activity of a desired recombinant polypeptide or protein. For example, the metabolic waste products may be detrimental to the growth or viability of the cell culture, may decrease the amount of recombinant polypeptide or protein produced, may alter the folding, stability, glycoslyation or other post-translational modification of the expressed polypeptide or protein, or may be detrimental to the cells and/or expression or activity of the recombinant polypeptide or protein in any number of other ways. Exemplary metabolic waste products include lactate, which is produced as a result of glucose metabolism, and ammonium, which is produced as a result of glutamine metabolism. One goal of the present invention is to slow production of, reduce or even eliminate metabolic waste products in mammalian cell cultures.

[0068] *Nucleic acid*: As used herein, the term "nucleic acid," in its broadest sense, refers to a compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. In some embodiments, "nucleic acid" refers to individual nucleic acid residues (*e.g.*, nucleotides and/or nucleosides). In some embodiments, "nucleic acid" refers to an oligonucleotide chain

comprising individual nucleic acid residues. As used herein, the terms "oligonucleotide" and "polynucleotide" can be used interchangeably. In some embodiments, "nucleic acid" encompasses RNA as well as single and/or double-stranded DNA and/or cDNA. Furthermore, the terms "nucleic acid," "DNA," "RNA," and/or similar terms include nucleic acid analogs, *i.e.*, analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention. The term "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and/or encode the same amino acid sequence. Nucleotide sequences that encode proteins and/or RNA may include introns. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, *etc.* Where appropriate, *e.g.*, in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, backbone modifications, *etc.* A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated. The term "nucleic acid segment" is used herein to refer to a nucleic acid sequence that is a portion of a longer nucleic acid sequence. In many embodiments, a nucleic acid segment comprises at least 3, 4, 5, 6, 7, 8, 9, 10, or more residues. In some embodiments, a nucleic acid is or comprises natural nucleosides (*e.g.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (*e.g.*, methylated bases); intercalated bases; modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (*e.g.*, phosphorothioates and 5'-*N*-phosphoramidite linkages). In some embodiments, the present invention is specifically directed to "unmodified nucleic acids," meaning nucleic acids (*e.g.*, polynucleotides and residues, including nucleotides and/or nucleosides) that have not been chemically modified in order to facilitate or achieve delivery.

[0069] *Osmolarity* and *Osmolality*: "Osmolality" is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution. The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of solution (1 mOsm/kg H_2O at 38°C is equivalent to an osmotic pressure of 19mm Hg). "Osmolarity," by contrast, refers to the number of solute particles dissolved in 1 liter of solution. When used herein, the abbreviation "mOsm" means "milliosmoles/kg solution".

[0070] *Perfusion process*: The term "perfusion process" as used herein refers to a method of culturing cells in which additional components are provided continuously or semi-continuously to the culture subsequent to the beginning of the culture process. The provided components typically comprise nutritional supplements for the cells which have been depleted during the culturing process. A portion of the cells and/or components in the medium are typically harvested on a continuous or semi-continuous basis and are optionally purified. Typically, a cell culture process involving a perfusion process is referred to as "perfusion culture." Typically, nutritional supplements are provided in a fresh medium during a perfusion process. In some embodiments, a fresh medium may be identical or similar to the base medium used in the cell culture process. In some embodiments, a fresh medium may be different than the base medium but containing desired nutritional supplements. In some embodiments, a fresh medium is a chemically-defined medium.

[0071] *Protein*: As used herein, the term "protein" refers to a polypeptide (*i.e.*, a string of at least two amino acids linked to one another by peptide bonds). Proteins may include moieties other than amino acids (*e.g.*, may be glycoproteins, proteoglycans, *etc.*) and/or may be otherwise processed or modified. Those of ordinary skill in the art will appreciate that a "protein" can be a complete polypeptide chain as produced by a cell (with or without a signal sequence), or can be a characteristic portion thereof. In some embodiments, a protein can sometimes include more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. In some embodiments, polypeptides may contain L-amino acids, D-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, *etc.* In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. The term "peptide" is generally used to refer to a polypeptide having a

length of less than about 100 amino acids, less than about 50 amino acids, less than 20 amino acids, or less than 10 amino acids. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

[0072] *Recombinant protein* and *Recombinant polypeptide*: These terms as used herein refer to a polypeptide expressed from a host cell, that has been genetically engineered to express that polypeptide. In some embodiments, a recombinant protein may be expressed in a host cell derived from an animal. In some embodiments, a recombinant protein may be expressed in a host cell derived from an insect. In some embodiments, a recombinant protein may be expressed in a host cell derived from a yeast. In some embodiments, a recombinant protein may be expressed in a host cell derived from a prokaryote. In some embodiments, a recombinant protein may be expressed in a host cell derived from an mammal. In some embodiments, a recombinant protein may be expressed in a host cell derived from a human. In some embodiments, the recombinantly expressed polypeptide may be identical or similar to a polypeptide that is normally expressed in the host cell. In some embodiments, the recombinantly expressed polypeptide may be foreign to the host cell, i.e. heterologous to peptides normally expressed in the host cell. Alternatively, in some embodiments the recombinantly expressed polypeptide can be a chimeric, in that portions of the polypeptide contain amino acid sequences that are identical or similar to polypeptides normally expressed in the host cell, while other portions are foreign to the host cell.

[0073] *Replacement enzyme*: As used herein, the term "replacement enzyme" refers to any enzyme that can act to replace at least in part the deficient or missing enzyme in a disease to be treated. In some embodiments, the term "replacement enzyme" refers to any enzyme that can act to replace at least in part the deficient or missing lysosomal enzyme in a lysosomal storage disease to be treated. In some embodiments, a replacement enzyme is capable of reducing accumulated materials in mammalian lysosomes or that can rescue or ameliorate one or more lysosomal storage disease symptoms. Replacement enzymes suitable for the invention include both wild-type or modified lysosomal enzymes and can be produced using recombinant and synthetic methods or purified from nature sources. A replacement enzyme can be a recombinant, synthetic, gene-activated or natural enzyme.

[0074] *Seeding*: The term "seeding" as used herein refers to the process of providing a cell culture to a bioreactor or another vessel for large scale cell culture production. In some embodiments a "seed culture" is used, in which the cells have been propagated in a smaller

cell culture vessel, i.e. Tissue-culture flask, Tissue-culture plate, Tissue-culture roller bottle, etc., prior to seeding. Alternatively, in some embodiments, the cells may have been frozen and thawed immediately prior to providing them to the bioreactor or vessel. The term refers to any number of cells, including a single cell.

[0075] *Subject:* As used herein, the term "subject" means any mammal, including humans. In certain embodiments of the present invention the subject is an adult, an adolescent or an infant. Also contemplated by the present invention are the administration of the pharmaceutical compositions and/or performance of the methods of treatment in-utero.

[0076] *Titer*: The term "titer" as used herein refers to the total amount of recombinantly expressed polypeptide or protein produced by a cell culture divided by a given amount of medium volume.

[0077] *Vector*: As used herein, "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it is associated. In some embodiment, vectors are capable of extra-chromosomal replication and/or expression of nucleic acids to which they are linked in a host cell such as a eukaryotic and/or prokaryotic cell. Vectors capable of directing the expression of operatively linked genes are referred to herein as "expression vectors."

[0078] *Viable cell density*: As used herein, the term "viable cell density" refers to the number of living cells per unit volume.

DETAILED DESCRIPTION OF THE INVENTION

[0079] The present invention provides, among other things, methods and compositions for large-scale production of recombinant I2S protein using suspension culture of mammalian cells in serum-free medium. In particular, the present invention uses mammalian cells that co-express a recombinant I2S protein and a formylglycine generating enzyme (FGE).

[0080] Various aspects of the invention are described in further detail in the following subsections. The use of subsections is not meant to limit the invention. Each subsection may apply to any aspect of the invention. In this application, the use of "or" means "and/or" unless stated otherwise.

Iduronate-2-sulfatase (I2S)

[0081] As used herein, an I2S protein is any protein or a portion of a protein that can substitute for at least partial activity of naturally-occurring Iduronate-2-sulfatase (I2S) protein or rescue one or more phenotypes or symptoms associated with I2S-deficiency. As used herein, the terms "an I2S enzyme" and "an I2S protein", and grammatical equivalents, are used inter-changeably.

[0082] Typically, the human I2S protein is produced as a precursor form. The precursor form of human I2S contains a signal peptide (amino acid residues 1-25 of the full length precursor), a pro-peptide (amino acid residues 26-33 of the full length precursor), and a chain (residues 34-550 of the full length precursor) that may be further processed into the 42 kDa chain (residues 34-455 of the full length precursor) and the 14 kDa chain (residues 446-550 of the full length precursor). Typically, the precursor form is also referred to as full-length precursor or full-length I2S protein, which contains 550 amino acids. The amino acid sequences of the mature form (SEQ ID NO:1) having the signal peptide removed and full-length precursor (SEQ ID NO:2) of a typical wild-type or naturally-occurring human I2S protein are shown in Table 1. The signal peptide is underlined. In addition, the amino acid sequences of human I2S protein isoform a and b precursor are also provided in Table 1, SEQ ID NO:3 and 4, respectively.

Table 1. Human Iduronate-2-sulfatase

Mature Form	SETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFA QQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSV GKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVD VLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKL YPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRK IRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYS NFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVEL VSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNP RELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFL ANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP(SEQ ID NO:1)
Full-Length Precursor (Isoform a)	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCY GDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSY WRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSS EKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSA SPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDI RQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLA NSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEKLF PYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELC REGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIK

	IMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQ GGDLFQLLMP(SEQ ID NO:2)
Isoform b Precursor	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCY GDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSY WRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSS EKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSA SPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDI RQREDVQALNISVPYGPIPVDFQEDQSSTGFRLKTSSTRKYK (SEQ ID NO:3)
Isoform c Precursor	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCY GDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSY WRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSS EKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSA SPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDI RQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLA NSTIIAFTSDHGFLMRTNT(SEQ ID No:4)

[0083] Thus, in some embodiments, an I2S enzyme is mature human I2S protein (SEQ ID NO:1). As disclosed herein, SEQ ID NO:1 represents the canonical amino acid sequence for the human I2S protein. In some embodiments, the I2S protein may be a splice isoform and/or variant of SEQ ID NO:1, resulting from transcription at an alternative start site within the 5' UTR of the I2S gene. In some embodiments, a suitable replacement enzyme may be a homologue or an analogue of mature human I2S protein. For example, a homologue or an analogue of mature human I2S protein may be a modified mature human I2S protein containing one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type or naturally-occurring I2S protein (e.g., SEQ ID NO:1), while retaining substantial I2S protein activity. Thus, in some embodiments, a replacement enzyme suitable for the present invention is substantially homologous to mature human I2S protein (SEQ ID NO:1). In some embodiments, a replacement enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:1. In some embodiments, a replacement enzyme suitable for the present invention is substantially identical to mature human I2S protein (SEQ ID NO:1). In some embodiments, a replacement enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:1. In some embodiments, a replacement enzyme suitable for the present invention contains a fragment or a portion of mature human I2S protein.

[0084] Alternatively, an I2S enzyme is full-length I2S protein. In some embodiments, an I2S enzyme may be a homologue or an analogue of full-length human I2S protein. For example, a homologue or an analogue of full-length human I2S protein may be a modified full-length human I2S protein containing one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type or naturally-occurring full-length I2S protein (e.g., SEQ ID NO:2), while retaining substantial I2S protein activity. Thus, in some embodiments, an I2S enzyme is substantially homologous to full-length human I2S protein (SEQ ID NO:2). In some embodiments, an I2S enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:2. In some embodiments, an I2S enzyme suitable for the present invention is substantially identical to SEQ ID NO:2. In some embodiments, an I2S enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:2. In some embodiments, an I2S enzyme suitable for the present invention contains a fragment or a portion of full-length human I2S protein. As used herein, a full-length I2S protein typically contains signal peptide sequence.

[0085] In some embodiments, an I2S enzyme suitable for the present invention is human I2S isoform a protein. In some embodiments, a suitable I2S enzyme may be a homologue or an analogue of human I2S isoform a protein. For example, a homologue or an analogue of human I2S isoform a protein may be a modified human I2S isoform a protein containing one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type or naturally-occurring human I2S isoform a protein (e.g., SEQ ID NO:3), while retaining substantial I2S protein activity. Thus, in some embodiments, an I2S enzyme is substantially homologous to human I2S isoform a protein (SEQ ID NO:3). In some embodiments, an I2S enzyme has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:3. In some embodiments, an I2S enzyme is substantially identical to SEQ ID NO:3. In some embodiments, an I2S enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:3. In some embodiments, an I2S enzyme suitable for the present invention contains a fragment

or a portion of human I2S isoform a protein. As used herein, a human I2S isoform a protein typically contains a signal peptide sequence.

[0086] In some embodiments, an I2S enzyme is human I2S isoform b protein. In some embodiments, an I2S enzyme may be a homologue or an analogue of human I2S isoform b protein. For example, a homologue or an analogue of human I2S isoform b protein may be a modified human I2S isoform b protein containing one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type or naturally-occurring human I2S isoform b protein (e.g., SEQ ID NO:4), while retaining substantial I2S protein activity. Thus, In some embodiments, an I2S enzyme is substantially homologous to human I2S isoform b protein (SEQ ID NO:4). In some embodiments, an I2S enzyme has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:4. In some embodiments, an I2S enzyme is substantially identical to SEQ ID NO:4. In some embodiments, an I2S enzyme has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:4. In some embodiments, an I2S enzyme suitable for the present invention contains a fragment or a portion of human I2S isoform b protein. As used herein, a human I2S isoform b protein typically contains a signal peptide sequence.

[0087] Homologues or analogues of human I2S proteins can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references that compile such methods. In some embodiments, conservative substitutions of amino acids include substitutions made among amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. In some embodiments, a "conservative amino acid substitution" refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made.

[0088] In some embodiments, I2S enzymes contain a moiety that binds to a receptor on the surface of cells to facilitate cellular uptake and/or lysosomal targeting. For example, such a receptor may be the cation-independent mannose-6-phosphate receptor (CI-MPR) which binds the mannose-6-phosphate (M6P) residues. In addition, the CI-MPR also binds other proteins including IGF-II. A suitable lysosomal targeting moiety can be IGF-I, IGF-II, RAP, p97, and variants, homologues or fragments thereof (e.g., including those peptide

having a sequence at least 70%, 75%, 80%, 85%, 90%, or 95% identical to a wild-type mature human IGF-I, IGF-II, RAP, p97 peptide sequence). In some embodiments, a suitable receptor that the M6P residues bind may be cation-dependent.

Formylglycine Generating Enzyme (FGE)

[0089] Typically, the enzyme activity of I2S is influenced by a post-translational modification of a conserved cysteine (e.g., corresponding to amino acid 59 of the mature human I2S (SEQ ID NO:1)) to formylglycine, which is also referred to as 2-amino-3-oxopropionic acid, or oxo-alanine. This post-translational modification generally occurs in the endoplasmic reticulum during protein synthesis and is catalyzed by Formylglycine Generating Enzyme (FGE). The specific enzyme activity of I2S is typically positively correlated with the extent to which the I2S has the formylglycine modification. For example, an I2S protein preparation that has a relatively high amount of formylglycine modification typically has a relatively high specific enzyme activity; whereas an I2S protein preparation that has a relatively low amount of formylglycine modification typically has a relatively low specific enzyme activity.

[0090] Thus, cells suitable for producing recombinant I2S protein according to the present invention typically express FGE protein. In some embodiments, suitable cells express an endogenous FGE protein. In some embodiments, suitable cells are engineered to express an exogenous or recombinant Formylglycine Generating Enzyme (FGE) in combination with recombinant I2S. In some embodiments, suitable cells are engineered to activate an endogenous FGE gene such that the expression level or activity of the FGE protein is increased.

[0091] Typically, the human FGE protein is produced as a precursor form. The precursor form of human FGE contains a signal peptide (amino acid residues 1-33 of the full length precursor) and a chain (residues 34-374 of the full length precursor). Typically, the precursor form is also referred to as full-length precursor or full-length FGE protein, which contains 374 amino acids. The amino acid sequences of the mature form (SEQ ID NO:5) having the signal peptide removed and full-length precursor (SEQ ID NO:6) of a typical wild-type or naturally-occurring human FGE protein are shown in Table 2.

Table 2. Human Formylglycine Generating Enzyme (FGE)

Mature Form	SQEAGTGAGAGSLAGSCGCGTPQRPGAHGSSAAAHRYSREANAPGPVPGERQLA HSKMVPIPAGVFTMGTDDPQIKQDGEAPARRVTIDAFYMDAYEVSNTEFEKFVN STGYLTEAEKFGDSFVFEGMLSEQVKTNIQQAVAAAPWWLPVKGANWRHPEGPD STILHRPDHPVLHVSWNDAVAYCTWAGKRLPTEAEWEYSCRGGLHNRLFPWGNK LQPKGQHYANIWQGEFPVTNTGEDGFQGTAPVDAFPPNGYGLYNIVGNAWEWTS DWWTVHHSVEETLNPKGPPSGKDRVKKGGSYMCHRSYCYRYRCAARSQNTPDSS ASNLGFRCAADRLPTMD (SEQ ID NO:5)
Full-Length Precursor	MAAPALGLVCGRCPELGLVLLLLLLSLLCGAAGSQEAGTGAGAGSLAGSCGCGT PQRPGAHGSSAAAHRYSREANAPGPVPGERQLAHSKMVPIPAGVFTMGTDDPQI KQDGEAPARRVTIDAFYMDAYEVSNTEFEKFVNSTGYLTEAEKFGDSFVFEGML SEQVKTNIQQAVAAAPWWLPVKGANWRHPEGPDSTILHRPDHPVLHVSWNDAVA YCTWAGKRLPTEAEWEYSCRGGLHNRLFPWGNKLQPKGQHYANIWQGEFPVTNT GEDGFQGTAPVDAFPPNGYGLYNIVGNAWEWTSDWWTVHHSVEETLNPKGPPSG KDRVKKGGSYMCHRSYCYRYRCAARSQNTPDSSASNLGFRCAADRLPTMD (SEQ ID NO:6)

[0092] Thus, in some embodiments, an FGE enzyme suitable for the present invention is mature human FGE protein (SEQ ID NO:5). In some embodiments, a suitable FGE enzyme may be a homologue or an analogue of mature human FGE protein. For example, a homologue or an analogue of mature human FGE protein may be a modified mature human FGE protein containing one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type or naturally-occurring FGE protein (e.g., SEQ ID NO:5), while retaining substantial FGE protein activity. Thus, in some embodiments, an FGE enzyme suitable for the present invention is substantially homologous to mature human FGE protein (SEQ ID NO:5). In some embodiments, an FGE enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:5. In some embodiments, an FGE enzyme suitable for the present invention is substantially identical to mature human FGE protein (SEQ ID NO:5). In some embodiments, an FGE enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:5. In some embodiments, an FGE enzyme suitable for the present invention contains a fragment or a portion of mature human FGE protein.

[0093] Alternatively, an FGE enzyme suitable for the present invention is full-length FGE protein. In some embodiments, an FGE enzyme may be a homologue or an analogue of full-length human FGE protein. For example, a homologue or an analogue of full-length human FGE protein may be a modified full-length human FGE protein containing one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type or

naturally-occurring full-length FGE protein (e.g., SEQ ID NO:6), while retaining substantial FGE protein activity. Thus, in some embodiments, an FGE enzyme suitable for the present invention is substantially homologous to full-length human FGE protein (SEQ ID NO:6). In some embodiments, an FGE enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:4. In some embodiments, an FGE enzyme suitable for the present invention is substantially identical to SEQ ID NO:6. In some embodiments, an FGE enzyme suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:6. In some embodiments, an FGE enzyme suitable for the present invention contains a fragment or a portion of full-length human FGE protein. As used herein, a full-length FGE protein typically contains signal peptide sequence.

[0094] Exemplary nucleic acid sequences and amino acid sequences encoding exemplary FGE proteins are disclosed US Publication No. 20040229250, the entire contents of which is incorporated herein by reference.

Host Cells

[0095] As used herein, the term "host cells" refers to cells that can be used to produce recombinant I2S enzyme. In particular, host cells are suitable for producing recombinant I2S enzyme at a large scale. In some embodiments, host cells are able to produce I2S enzyme in an amount of or greater than about 5 picogram/cell/day (e.g., greater than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 picogram/cell/day). In some embodiments, host cells are able to produce I2S enzyme in an amount ranging from about 5-100 picogram/cell/day (e.g., about 5-90 picogram/cell/day, about 5-80 picogram/cell/day, about 5-70 picogram/cell/day, about 5-60 picogram/cell/day, about 5-50 picogram/cell/day, about 5-40 picogram/cell/day, about 5-30 picogram/cell/day, about 10-90 picogram/cell/day, about 10-80 picogram/cell/day, about 10-70 picogram/cell/day, about 10-60 picogram/cell/day, about 10-50 picogram/cell/day, about 10-40 picogram/cell/day, about 10-30 picogram/cell/day, about 20-90 picogram/cell/day, about 20-80 picogram/cell/day, about 20-70 picogram/cell/day, about 20-60 picogram/cell/day, about 20-50 picogram/cell/day,

about 20-40 picogram/cell/day, about 20-30 picogram/cell/day). In some embodiments, a suitable host cell is not a endosomal acidification-deficient cell.

[0096] Suitable host cells can be derived from a variety of organisms, including, but not limited to, mammals, plants, birds (e.g., avian systems), insects, yeast, and bacteria. Insome embodiments, host cells are mammalian cells. Any mammalian cell susceptible to cell culture, and to expression of polypeptides, may be utilized in accordance with the present invention as a host cell. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include human embryonic kidney 293 cells (HEK293), HeLa cells; BALB/c mouse myeloma line (NSO/l, ECACC No: 85110503); human retinoblasts (PER.C6 (CruCell, Leiden, The Netherlands)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human fibrosarcomacell line (e.g., HT-1080); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2), human cell line CAP and AGE1.HN, and Glycotope's panel.

[0097] Additionally, any number of available hybridoma cell lines may be utilized in accordance with the present invention. One skilled in the art will appreciate that hybridoma cell lines might have different nutrition requirements and/or might require different culture conditions for optimal growth and polypeptide or protein expression, and will be able to modify conditions as needed.

[0098] In some embodiments, host cells are non-mammalian cells. Non-limiting examples of non-mammalian host cells suitable for the present invention include cells and cell lines derived from *Pichia pastoris*, *Pichia methanolica*, *Pichia angusta*, *Schizosacccharomyces pombe*, *Saccharomyces cerevisiae,* and *Yarrowia lipolytica* for yeast; *Sodoptera frugiperda*, *Trichoplusis ni*, *Drosophila melangoster* and *Manduca sexta* for

insects; and *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis*, *Bacillus lichenifonnis*, *Bacteroides fragilis*, *Clostridia perfringens*, *Clostridia difficile* for bacteria; and *Xenopus Laevis* from amphibian.

Vectors and Nucleic Acid Constructs

[0099] Various nucleic acid constructs can be used to express I2S and/or FGE enzyme described herein in host cells. A suitable vector construct typically includes, in addition to I2S and/or FGE protein-encoding sequences (also referred to as I2S or FGE transgene), regulatory sequences, gene control sequences, promoters, non-coding sequences and/or other appropriate sequences for expression of the protein and, optionally, for replication of the construct. Typically, the coding region is operably linked with one or more of these nucleic acid components.

[0100] "Regulatory sequences" typically refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, enhancers, 5' untranslated sequences, translation leader sequences, introns, and 3' untranslated sequences such as polyadenylation recognition sequences. Sometimes, "regulatory sequences" are also referred to as "gene control sequences."

[0101] "Promoter" typically refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions.

[0102] The "3' non-coding sequences" typically refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

[0103] The "translation leader sequence" or "5' non-coding sequences" typically refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

[0104] Typically, the term "operatively linked" refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operatively linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operatively linked to regulatory sequences in sense or antisense orientation.

[0105] The coding region of a transgene may include one or more silent mutations to optimize codon usage for a particular cell type. For example, the codons of an I2S transgene may be optimized for expression in a vertebrate cell. In some embodiments, the codons of an I2S transgene may be optimized for expression in a mammalian cell. In some embodiments, the codons of an I2S transgene may be optimized for expression in a human cell.

[0106] Optionally, a construct may contain additional components such as one or more of the following: a splice site, an enhancer sequence, a selectable marker gene under the control of an appropriate promoter, an amplifiable marker gene under the control of an appropriate promoter, and a matrix attachment region (MAR) or other element known in the art that enhances expression of the region where it is inserted.

[0107] Once transfected or transduced into host cells, a suitable vector can express extrachromosomally (episomally) or integrate into the host cell's genome.

[0108] In some embodiments, a DNA construct that integrates into the cell's genome, it need include only the transgene nucleic acid sequences. In that case, the express of the

transgene is typically controlled by the regulatory sequences at the integration site. Optionally, it can include additional various regulatory sequences described herein.

Culture Medium

[0109] The term "medium" and "culture medium" as used herein refers to a general class of solution containing nutrients suitable for maintaining and/or growing cells *in vitro*. Typically, medium solutions provide, without limitation, essential and nonessential amino acids, vitamins, energy sources, lipids, and trace elements required by the cell for at least minimal growth and/or survival. In other embodiments, the medium may contain an amino acid(s) derived from any source or method known in the art, including, but not limited to, an amino acid(s) derived either from single amino acid addition(s) or from a peptone or protein hydrolysate addition(s) (including animal or plant source(s)). Vitamins such as, but not limited to, Biotin, Pantothenate, Choline Chloride, Folic Acid, Myo-Inositol, Niacinamide, Pyridoxine, Riboflavin, Vitamin B12, Thiamine, Putrescine and/or combinations thereof. Salts such as, but not limited to, $CaCl_2$, KCl, $MgCl_2$, NaCl, Sodium Phosphate Monobasic, Sodium Phosphate Dibasic, Sodium Selenite, $CuSO_4$, $ZnCl_2$ and/or combinations thereof. Fatty acids such as, but not limited to, Arachidonic Acid, Linoleic Acid, Oleic Acid, Lauric Acid, Myristic Acid, as well as Methyl-beta-Cyclodextrin and/or combinations thereof). In some embodiments, medium comprises additional components such as glucose, glutamine, Na-pyruvate, insulin or ethanolamine, a protective agent such as Pluronic F68. In some embodiments, the medium may also contain components that enhance growth and/or survival above the minimal rate, including hormones and growth factors. Medium may also comprise one or more buffering agents. The buffering agents may be designed and/or selected to maintain the culture at a particular pH (*e.g.*, a physiological pH, (*e.g.*, pH 6.8 to pH 7.4)). A variety of buffers suitable for culturing cells are known in the art and may be used in the methods. Suitable buffers (*e.g.*, bicarbonate buffers, HEPES buffer, Good's buffers, *etc.*) are those that have the capacity and efficiency for maintaining physiological pH despite changes in carbon dioxide concentration associated with cellular respiration. The solution is preferably formulated to a pH and salt concentration optimal for cell survival and proliferation.

[0110] In some embodiments, medium may be a chemically defined medium. As used herein, the term "chemically-defined nutrient medium" refers to a medium of which

substantially all of the chemical components are known. In some embodiments, a chemically defined nutrient medium is free of animal-derived components. In some cases, a chemically-defined medium comprises one or more proteins (*e.g.*, protein growth factors or cytokines.) In some cases, a chemically-defined nutrient medium comprises one or more protein hydrolysates. In other cases, a chemically-defined nutrient medium is a protein-free media, i.e., a serum-free media that contains no proteins, hydrolysates or components of unknown composition.

[0111] Typically, a chemically defined medium can be prepared by combining various individual components such as, for example, essential and nonessential amino acids, vitamins, energy sources, lipids, salts, buffering agents, and trace elements, at predetermined weight or molar percentages or ratios. Exemplary serum-free, in particular, chemically-defined media are described in US Pub. No. 2006/0148074, the disclosure of which is hereby incorporated by reference.

[0112] In some embodiments, a chemically defined medium suitable for the present invention is a commercially available medium such as, but not limited to, Dulbecco's Modified Eagle's Medium (DMEM), DMEM F12 (1:1), Ham's Nutrient mixture F-10, Roswell Park Memorial Institute Medium (RPMI), MCDB 131, William's Medium E, CD CHO medium (Invitrogen®), CD 293 medium (Invitrogen®), EX-Cell CDCHO, Ex-Cell CDCHO Fusion, CD-OptiCHO, CD-FortiCHO, CDM4CHO, CD1000, BalanCD-CHO, IS-CHO-CD, CD Hybridoma, CD-DG44. In some embodiments, a chemically defined medium suitable for the present invention is a mixture of one or more commercially available chemically defined mediums. In various embodiments, a suitable medium is a mixture of two, three, four, five, six, seven, eight, nine, ten, or more commercially available chemically defined media. In some embodiments, each individual commercially available chemically defined medium (e.g., such as those described herein) constitutes, by weight, 1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more, of the mixture. Ratios between each individual component medium may be determined by relative weight percentage present in the mixture.

[0113] In some embodiments, a chemically defined medium may be supplemented by one or more animal derived components. Such animal derived components include, but are not limited to, fetal calf serum, horse serum, goat serum, donkey serum, human serum, and

serum derived proteins such as albumins (e.g., bovine serum albumin or human serum albumin).

Redox-modulators

[0114] In some embodiments, a suitable medium contains one or more redox-modulators. Without wishing to be bound by particular theory, it is contemplated that redox-modulators may improve the production and/or activity of I2S, leading to recombinant I2S compositions having high levels of active enzyme. As used herein, a "redox-modulator" is a molecule (*e.g.*, small-molecule, polypeptide, *etc.*) that influences the likelihood that a constituent in a mixture will acquire electrons and thereby be reduced. A redox-modulator may increase or decrease the likelihood that a constituent in the mixture will acquire electrons and thereby be reduced. In some embodiments, a redox-modulator may already be present in a medium, *e.g.*, when a chemically-defined medium is obtained from a commercially available source, or may be provided as an additive to the medium. In some cases, a medium according to the invention contains two or more redox-modulators. Non-limiting examples of redox-modulators include glutathione, glucose-6-phosphate, carnosine, carnosol, sulforaphane, tocopherol, ascorbate, dehydroascorbate, selenium, 2-mercaptoenthanol, N-acetylcysteine, cysteine, riboflavin, niacin, folate, flavin adenine dinucleotide (FAD), dithiothreitol and nicotinamide adenine dinucleotide phosphate (NADP). Other appropriate redox-modulators will be apparent to the skilled artisan.

[0115] In some embodiments, cysteine is added to, or present in, a medium of the invention. Cysteine may be present at various concentrations. In some embodiments, the concentration of cysteine in the medium is in a range of about 0.1 mg/L to about 10 mg/L, about 1 mg/L to about 25 mg/L, about 10 mg/L to about 50 mg/L, about 25 mg/L to about 65 mg/L, about 10 mg/L to about 100 mg/L, or about 25 mg/L to about 250 mg/L. In some embodiments, the cysteine is at a concentration ranging from about 0.1 mg/L to about 65 mg/L (e.g., 1-50 mg/L, 1-40 mg/L, 1-30 mg/l, 1-20 mg/L, 1-10 mg/L). In some cases, the concentration of cysteine in the medium is up to about 0.1 mg/L, about 1 mg/L, about 5 mg/L, about 10 mg/L, about 20 mg/L, about 25 mg/L, about 50 mg/L, about 65 mg/L, about 75 mg/L, about 100 mg/L, or more.

[0116] In some embodiments, 2-mercaptoenthanol is added to, or present in, a medium of the invention. Various concentrations may be used. In some embodiments, the concentration of 2-mercaptoenthanol is in a range of about 0.1 nM to about 0.001 mM, about 0.001 mM to about 0.01 mM, about 0.001 mM to about 0.1 mM, about 0.01 mM to about 0.1 mM, about 0.01 mM to about 1 mM. In some cases, the concentration of 2-mercaptoenthanol in up to about 0.1 nM, about 0.001 mM, about 0.01 mM, about 0.1 mM, about 1 mM or more. In some embodiments, the 2-mercaptoenthanol is at a concentration ranging from about 0.001 mM to about 0.01 mM (e.g., about 0.001-0.008 mM, about 0.001-0.007 mM, about 0.001-0.006 mM, about 0.001-0.005 mM, about 0.001-0.004 mM, about 0.001-0.003 mM, about 0.001-0.002 mM).

[0117] In some embodiments, N-acetylcysteine is added to, or present in, a medium of the invention. Various concentrations may be used. In some embodiments, the concentration of the N-acetylcysteine may be in a range of about 0.1 mM to about 1 mM, about 1 mM to about 10 mM, about 3 mM to about 9 mM, about 1 mM to about 50 mM, or about 10 mM to about 50 mM. In some embodiments, the N-acetylcysteine is at a concentration ranging from about 3 mM to about 9 mM (e.g., about 3-8 mM, about 3-7 mM, about 3-6 mM, about 3-5 mM, about 3-4 mM). In some embodiments, the concentration of the N-acetylcysteine may up to about 0.1 mM, about 1 mM, about 3 mM, about 9 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, or more.

Growth-modulators

[0118] In some embodiments, a medium may contain one or more growth-modulators to improve the production of I2S. As used herein, the term "growth-modulator" refers to a molecule that affects the growth of a cell. A growth-modulator can increase cell growth by, *e.g.*, enhancing or inducing cell proliferation, cell cycle progression, or decrease cell growth by, *e.g.*, promoting cell cycle arrest. While commercially available mediums often comprise a multitude of different growth-modulators, in some cases it is desirable to provide additional growth modulators to the nutrient medium. Therefore, in some embodiments, one or more growth-modulators are added to the medium.

[0119] In some cases, a growth-modulator suitable for the invention includes hypoxanthine. In some embodiments, hypoxanthine is at a concentration in a range of about

0.01 mM to about 0.1 mM, about 0.1 mM to about 1 mM, about 0.1 mM to about 10 mM, about 1 mM to about 10 mM, about 0.1 mM to about 100 mM. In some embodiments, the hypoxanthine is at a concentration ranging from about 0.1 mM to about 10 mM (e.g., about 0.1-9 mM, about 0.1-8 mM, about 0.1-7 mM, about 0.1-6 mM, about 0.1-5 mM, about 0.1-4 mM, about 0.1-3 mM, about 0.1-2 mM, about 0.1-1 mM). In some cases, hypoxanthine is at a concentration of about 0.01 mM, about 0.1 mM, about 1 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM or more.

[0120] In some cases, a growth-modulator suitable for the invention includes thymidine. In some embodiments, the thymidine is at a concentration in a range of about 0.01 mM to about 0.1 mM, about 0.1 mM to about 1 mM, about 0.1 mM to about 10 mM, about 1 mM to about 10 mM, about 0.1 mM to about 100 mM, about 1 mM to about 100 mM. In some embodiments, the thymidine is at a concentration ranging from about 1 mM to about 100 mM (e.g., about 1-90 mM, about 1-80 mM, about 1-70 mM, about 1-60 mM, about 1-50 mM, about 1-40 mM, about 1-30 mM, about 1-20 mM, about 1-10 mM). In some embodiments, thymidine is at a concentration of about 0.01 mM, about 0.1 mM, about 1 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM or more.

Culture Conditions

[0121] The present invention provides a method of producing recombinant I2S at a large scale. Typical large-scale procedures for producing a recombinant polypeptide of interest include batch cultures and fed-batch cultures. Batch culture processes traditionally comprise inoculating a large-scale production culture with a seed culture of a particular cell density, growing the cells under conditions (e.g., suitable culture medium, pH, and temperature) conducive to cell growth, viability, and/or productivity, harvesting the culture when the cells reach a specified cell density, and purifying the expressed polypeptide. Fed-batch culture procedures include an additional step or steps of supplementing the batch culture with nutrients and other components that are consumed during the growth of the cells. In some embodiments, a large-scale production method according to the present invention uses a fed-batch culture system.

Culture Initiation

[0122] Typically, a desired cell expressing I2S protein is first propagated in an initial culture by any of the variety of methods well-known to one of ordinary skill in the art. The cell is typically propagated by growing it at a temperature and in a medium that is conducive to the survival, growth and viability of the cell. The initial culture volume can be of any size, but is often smaller than the culture volume of the production bioreactor used in the final production, and frequently cells are passaged several times of increasing culture volume prior to seeding the production bioreactor. The cell culture can be agitated or shaken to increase oxygenation of the medium and dispersion of nutrients to the cells. Alternatively or additionally, special sparging devices that are well known in the art can be used to increase and control oxygenation of the culture.

[0123] The starting cell density can be chosen by one of ordinary skill in the art. In accordance with the present invention, the starting cell density can be as low as a single cell per culture volume. In some embodiments, starting cell densities can range from about 1 X 10^2 viable cells per mL to about 1 X 10^3, 1 X 10^4, 1 X 10^5 viable cells per mL and higher.

[0124] Initial and intermediate cell cultures may be grown to any desired density before seeding the next intermediate or final production bioreactor. In some embodiments, final viability before seeding the production bioreactor is greater than about 70%, 75%, 80%, 85%, 90%, 95%, or more. The cells may be removed from the supernatant, for example, by low-speed centrifugation. It may also be desirable to wash the removed cells with a medium before seeding the next bioreactor to remove any unwanted metabolic waste products or medium components. The medium may be the medium in which the cells were previously grown or it may be a different medium or a washing solution selected by the practitioner of the present invention.

[0125] The cells may then be diluted to an appropriate density for seeding the production bioreactor. In some embodiments, the cells are diluted into the same medium that will be used in the production bioreactor. Alternatively, the cells can be diluted into another medium or solution, depending on the needs and desires of the practitioner of the present invention or to accommodate particular requirements of the cells themselves, for example, if they are to be stored for a short period of time prior to seeding the production bioreactor.

Growth Phase

[0126] Typically, once the production bioreactor has been seeded as described above, the cell culture is maintained in the initial growth phase under conditions conducive to the survival, growth and viability of the cell culture. In accordance with the present invention, the production bioreactor can be any volume that is appropriate for large-scale production of proteins. See the "Bioreactor" subsection below.

[0127] The temperature of the cell culture in the growth phase is selected based primarily on the range of temperatures at which the cell culture remains viable. The temperature of the growth phase may be maintained at a single, constant temperature, or within a range of temperatures. For example, the temperature may be steadily increased or decreased during the growth phase. In general, most mammalian cells grow well within a range of about 25 °C to 42 °C (e.g., 30 °C to 40 °C, about 30 °C to 37 °C, about 35 °C to 40 °C). In some embodiments, the mammalian cells are cultured at a temperature ranging from about 30-37 °C (e.g., about 31-37 °C, about 32-37 °C, about 33-37 °C, about 34-37 °C, about 35-37 °C, about 36-37 °C). Typically, during the growth phase, cells grow at about 28 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, about 37 °C., about 38 °C, about 39 °C, about 40 °C.

[0128] The cells may be grown during the initial growth phase for a greater or lesser amount of time, depending on the needs of the practitioner and the requirement of the cells themselves. In one embodiment, the cells are grown for a period of time sufficient to achieve a viable cell density that is a given percentage of the maximal viable cell density that the cells would eventually reach if allowed to grow undisturbed. For example, the cells may be grown for a period of time sufficient to achieve a desired viable cell density of 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal viable cell density.

[0129] In some embodiment, the cells are allowed to grow for a defined period of time. For example, depending on the starting concentration of the cell culture, the temperature at which the cells are grown, and the intrinsic growth rate of the cells, the cells may be grown for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more days. In some cases, the cells may be allowed to grow for a month or more.

[0130] In some embodiments, the cells are allowed to grow to a desired viable cell density. For example, a desired viable cell density by the end of growth phase is greater than about 1.0 X 10^6 viable cells/mL, 1.5 X 10^6 viable cells/mL, 2.0 X 10^6 viable cells/mL, 2.5 X 10^6 viable cells/mL, 5 X 10^6 viable cells/mL, 10 X 10^6 viable cells/mL, 20X 10^6 viable cells/mL, 30 X 10^6 viable cells/mL, 40 X 10^6 viable cells/mL, or 50 X 10^6 viable cells/mL.

[0131] The cell culture may be agitated or shaken during the initial culture phase in order to increase oxygenation and dispersion of nutrients to the cells. In accordance with the present invention, one of ordinary skill in the art will understand that it can be beneficial to control or regulate certain internal conditions of the bioreactor during the initial growth phase, including but not limited to pH, temperature, oxygenation, etc. For example, pH can be controlled by supplying an appropriate amount of acid or base and oxygenation can be controlled with sparging devices that are well known in the art. In some embodiments, a desired pH for the growth phase ranges from about 6.8 – 7.5 (e.g., about 6.9-7.4, about 6.9-7.3, about 6.95-7.3, about 6.95-7.25, about 7.0-7.3, about 7.0-7.25, about 7.0-7.2, about 7.0-7.15, about 7.05-7.3, about 7.05-7.25, about 7.05-7.15, about 7.05-7.20, about 7.10-7.3, about 7.10-7.25, about 7.10-7.20, about 7.10-7.15). In some embodiments, a desired pH for the growth phase is about 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, 7.4, 7.45, or 7.5.

Transition phase

[0132] In some embodiments, when the cells are ready for the production phase, the culture conditions may be changed to maximize the production of the recombinant protein of interest. Such culture condition change typically takes place in a transition phase. In some embodiments, such change may be a shift in one or more of a number of culture conditions including, but not limited to, temperature, pH, osmolarity and medium. In one embodiment, the pH of the culture is shifted. For example, the pH of the medium may be increased or decrease from growth phase to the production phase. In some embodiments, this change in pH is rapid. In some embodiments, this change in pH occurs slowly over a prolonged period of time. In some embodiments, the change in pH regulated by the addition of sodium biocarbonate. In some embodiments, the change in pH is initiated at the start of the transition phase and is maintained during the subsequent production phase.

[0133] In one embodiments, the glucose concentration of the cell culture medium is shifted. According to this embodiment, upon initiation of the transition phase, the glucose concentration within the cell culture is adjusted to a rate higher than 7.5 mM.

[0134] In some embodiments, the temperature is shifted up or down from the growth phase to production phase. For example, the temperature may be shifted up or down from growth phase to the production phase by about 0.1 °C, 0.2 °C, 0.3 °C, 0.4 °C, 0.5 °C, 1.0 °C, 1.5 °C, 2.0 °C, 2.5 °C, 3.0 °C, 3.5 °C, 4.0 °C, 4.5 °C, 5.0 °C, or more.

Production Phase

[0135] In accordance with the present invention, once the cell culture reaches a desired cell density and viability, with or without a transition phase, the cell culture is maintained for a subsequent production phase under culture conditions conducive to the survival and viability of the cell culture and appropriate for expression of I2S and/or FGE protein at commercially adequate levels.

[0136] In some embodiments, during the production phase, the culture is maintained at a temperature or temperature range that is lower than the temperature or temperature range of the growth phase. For example, during the production phase, cells may express recombinant I2S and/or FGE proteins well within a range of about 25 °C to 35 °C (e.g., about 28 °C to 35 °C, about 30 °C to 35 °C. about 32 °C to 35 °C). In some embodiments, during the production phase, cells may express recombinant I2S and/or FGE proteins well at a temperature of about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, about 37 °C. In other embodiments, during the production phase, the culture is maintained at a temperature or temperature range that is higher than the temperature or temperature range of the growth phase.

[0137] Additionally or alternatively, during the production phase, the culture is maintained at a pH or pH range that is different (lower or higher) than the pH or pH range of the growth phase. In some embodiments, the medium for the production phase has a pH ranging from about 6.8 – 7.5 (e.g., about 6.9-7.4, about 6.9-7.3, about 6.95-7.3, about 6.95-7.25, about 7.0-7.3, about 7.0-7.25, about 7.0-7.2, about 7.0-7.15, about 7.05-7.3, about 7.05-7.25, about 7.05-7.15, about 7.05-7.20, about 7.10-7.3, about 7.10-7.25, about 7.10-7.20,

about 7.10-7.15). In some embodiments, the medium has a pH of about 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, 7.4, 7.45, or 7.5.

[0138] In some embodiments, the cells may be maintained within a desired viable cell density range throughout the production. For example, during the production phase of the cell culture, a desired viable cell density may range from about 1.0-50 X 10^6 viable cells/mL during the production phase (e.g., about 1.0-40 X 10^6 viable cells/mL, about 1.0-30 X 10^6 viable cells/mL, about 1.0-20 X 10^6 viable cells/mL, about 1.0-10 X 10^6 viable cells/mL, about 1.0-5 X 10^6 viable cells/mL, about 1.0-4.5 X 10^6 viable cells/mL, about 1.0-4 X 10^6 viable cells/mL, about 1.0-3.5 X 10^6 viable cells/mL, about 1.0-3 X 10^6 viable cells/mL, about 1.0-2.5 X 10^6 viable cells/mL, about 1.0-2.0 X 10^6 viable cells/mL, about 1.0-1.5 X 10^6 viable cells/mL, about 1.5-10 X 10^6 viable cells/mL, about 1.5-5 X 10^6 viable cells/mL, about 1.5-4.5 X 10^6 viable cells/mL, about 1.5-4 X 10^6 viable cells/mL, about 1.5-3.5 X 10^6 viable cells/mL, about 1.5-3.0 X 10^6 viable cells/mL, about 1.5-2.5 X 10^6 viable cells/mL, about 1.5-2.0 X 10^6 viable cells/mL).

[0139] In some embodiments, the cells may be maintained for a period of time sufficient to achieve a viable cell density of 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 percent of maximal viable cell density. In some cases, it may be desirable to allow the viable cell density to reach a maximum. In some embodiments, it may be desirable to allow the viable cell density to reach a maximum and then allow the viable cell density to decline to some level before harvesting the culture. In some embodiments, the total viability at the end of the production phase is less than about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%.

[0140] In some embodiments, the cells are allowed to grow for a defined period of time during the production phase. For example, depending on the concentration of the cell culture at the start of the subsequent growth phase, the temperature at which the cells are grown, and the intrinsic growth rate of the cells, the cells may be grown for about 5-90 days (e.g., about 5-80 days, about 5-70 days, about 5-60 days, about 5-50 days, about 5-40, about 5-30 days, about 5-20 days, about 5-15 days, about 5-10 days, about 10-90 days, about 10-80 days, about 10-70 days, about 10-60 days, about 10-50 days, about 10-40 days, about 10-30 days, about 10-20 days, about 15-90 days, about 15-80 days, about 15-70 days, about 15-60 days, about 15-50 days, about 15-40 days, about 15-30 days). In some embodiments, the

production phase is lasted for about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 days.

[0141] In some embodiments, the cells are maintained in the production phase until the titer to the recombinant I2S protein reaches a maximum. In other embodiments, the culture may be harvested prior to this point. For example, in some embodiments, the cells are maintained in the production phase until the titer to the recombinant I2S protein reaches a desired titer. Thus, a desired average harvest titer to the recombinant I2S protein may be of at least 6 mg per liter per day (mg/L/day) (e.g., at least 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, or 500 mg/L/day, or more). In some embodiments, a desired average harvest titer to the recombinant I2S protein may range from about 6-500 mg/L/day (e.g., about 6-400 mg/L/day, about 6-300 mg/L/day, about 6-200 mg/L/day, about 6-100 mg/L/day, about 6-90 mg/L/day, about 6-80 mg/L/day, about 6-70 mg/L/day, about 6-60 mg/L/day, about 6-50 mg/L/day, about 6-40 mg/L/day, about 6-30 mg/L/day, about 10-500 mg/L/day, about 10-400 mg/L/day, about 10-300 mg/L/day, about 10-200 mg/L/day, about 10-100 mg/L/day, about 10-90 mg/L/day, about 10-80 mg/L/day, about 10-70 mg/L/day, about 10-60 mg/L/day, about 10-50 mg/L/day, about 10-40 mg/L/day, about 10-30 mg/L/day, about 20-500 mg/L/day, about 20-400 mg/L/day, about 20-300 mg/L/day, about 20-200 mg/L/day, about 20-100 mg/L/day, about 20-90 mg/L/day, about 20-80 mg/L/day, about 20-70 mg/L/day, about 20-60 mg/L/day, about 20-50 mg/L/day, about 20-40 mg/L/day, about 20-30 mg/L/day).

[0142] Additionally or alternatively, the cells are maintained in the production phase under conditions such that the produced recombinant I2S protein reach a desired C_α-formylglycine (FGly) conversion percentage. In some embodiments, the produced recombinant I2S protein contains at least about 70% (e.g., at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100%) conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_α-formylglycine (FGly).

[0143] Additionally or alternatively, the cells are maintained in the production phase under conditions such that the produced recombinant I2S protein reach a desired enzymatic activity. As can be appreciated by one skilled in the art, the enzymatic activity of recombinant I2S protein may be measured by various *in vitro* and *in vivo* assays. In some embodiments, a desired enzymatic activity, as measured by *in vitro* sulfate release activity assay using heparin disaccharide as substrate, of the produced recombinant I2S protein is at

least about 20 U/mg, 30 U/mg, 40 U/mg, 50 U/mg, 60 U/mg, 70 U/mg, 80 U/mg, 90 U/mg, or 100 U/mg. In some embodiments, a desired enzymatic activity, as measured by *in vitro* sulfate release activity assay using heparin disaccharide as substrate, of the produced recombinant I2S protein ranges from about 20-100 U/mg (e.g., about 20-90 U/mg, about 20-80 U/mg, about 20-70 U/mg, about 20-60 U/mg, about 20-50 U/mg, about 20-40 U/mg, about 20-30 U/mg, about 30-100 U/mg, about 30-90 U/mg, about 30-80 U/mg, about 30-70 U/mg, about 30-60 U/mg, about 30-50 U/mg, about 30-40 U/mg, about 40-100 U/mg, about 40-90 U/mg, about 40-80 U/mg, about 40-70 U/mg, about 40-60 U/mg, about 40-50 U/mg). Exemplary conditions for performing *in vitro* sulfate release activity assay using heparin disaccharide as substrate are provided below. Typically, this assay measures the ability of I2S to release sulfate ions from a naturally derived substrate, heparin diasaccharide. The released sulfate may be quantified by ion chromatography. In some cases, ion chromatography is equipped with a conductivity detector. As a non-limiting example, samples are first buffer exchanged to 10 mM Na acetate, pH 6 to remove inhibition by phosphate ions in the formulation buffer. Samples are then diluted to 0.075 mg/ml with reaction buffer (10 mM Na acetate, pH 4.4) and incubated for 2 hrs at 37°C with heparin disaccharide at an enzyme to substrate ratio of 0.3 μg I2S/100 μg substrate in a 30 μL reaction volume. The reaction is then stopped by heating the samples at 100°C for 3 min. The analysis is carried out using a Dionex IonPac AS18 analytical column with an IonPac AG18 guard column. An isocratic method is used with 30 mM potassium hydroxide at 1.0 mL/min for 15 minutes. The amount of sulfate released by the I2S sample is calculated from the linear regression analysis of sulfate standards in the range of 1.7 to 16.0 nmoles. The reportable value is expressed as Units per mg protein, where 1 unit is defined as 1 μmoles of sulfate released per hour and the protein concentration is determined by A280 measurements.

[0144] In some embodiments, the enzymatic activity of recombinant I2S protein may also be determined using various other methods known in the art such as, for example, 4-MUF assay which measures hydrolysis of 4-methylumbelliferyl-sulfate to sulfate and naturally fluorescent 4-methylumbelliferone (4-MUF). In some embodiments, a desired enzymatic activity, as measured by *in vitro* 4-MUF assay, of the produced recombinant I2S protein is at least about 2 U/mg, 4 U/mg, 6 U/mg, 8 U/mg, 10 U/mg, 12 U/mg, 14 U/mg, 16 U/mg, 18 U/mg, or 20 U/mg. In some embodiments, a desired enzymatic activity, as measured by *in vitro* 4-MUF assay, of the produced recombinant I2S protein ranges from about 0-50 U/mg (e.g., about 0-40 U/mg, about 0-30 U/mg, about 0-20 U/mg, about 0-10

U/mg, about 2-50 U/mg, about 2-40 U/mg, about 2-30 U/mg, about 2-20 U/mg, about 2-10 U/mg, about 4-50 U/mg, about 4-40 U/mg, about 4-30 U/mg, about 4-20 U/mg, about 4-10 U/mg, about 6-50 U/mg, about 6-40 U/mg, about 6-30 U/mg, about 6-20 U/mg, about 6-10 U/mg). Exemplary conditions for performing *in vitro* 4-MUF assay are provided below. Typically, a 4-MUF assay measures the ability of an I2S protein to hydrolyze 4-methylumbelliferyl-sulfate (4-MUF-SO_4) to sulfate and naturally fluorescent 4-methylumbelliferone (4-MUF). One milliunit of activity is defined as the quantity of enzyme required to convert one nanomole of 4-MUF-SO_4 to 4-MUF in one minute at 37°C. Typically, the mean fluorescence units (MFU) generated by I2S test samples with known activity can be used to generate a standard curve, which can be used to calculate the enzymatic activity of a sample of interest.

[0145] In some embodiments, it may be beneficial or necessary to supplement the cell culture during the production phase with nutrients or other medium components that have been depleted or metabolized by the cells. For example, it might be advantageous to supplement the cell culture with nutrients or other medium components observed to have been depleted during the cell culture. Alternatively or additionally, it may be beneficial or necessary to supplement the cell culture prior to the production phase. As non-limiting examples, it may be beneficial or necessary to supplement the cell culture with redox-modulators, growth modulators (e.g., hormones and/or other growth factors), particular ions (such as sodium, chloride, calcium, magnesium, and phosphate), buffers, vitamins, nucleosides or nucleotides, trace elements (inorganic compounds usually present at very low final concentrations), amino acids, lipids, or glucose or other energy source.

[0146] These supplementary components may all be added to the cell culture at one time, or they may be provided to the cell culture in a series of additions. In some embodiments, the supplementary components are provided to the cell culture at multiple times in proportional amounts. In other embodiments, the cell culture is fed continually with these supplementary components. Typically, this process is known as perfusion and a cell culture involving perfusion is known as "perfusion culture." As used herein, the term "perfusion culture" refers to a method of culturing cells in which additional components are provided continuously or semi-continuously to the culture subsequent to the beginning of the culture process. A portion of the cells and/or components in the medium are typically harvested on a continuous or semi-continuous basis and are optionally purified.

[0147] In some embodiments, the medium is continuously exchanged by a perfusion process during the production phase. Typically, volume of fresh medium relative to working volume of reactor per day (VVD) is defined as perfusion rate. Various perfusion rates may be used in according to the present invention. In some embodiments, a perfusion process has a perfusion rate such that the total volume added to the cell culture be kept to a minimal amount. In some embodiments, the perfusion process has a perfusion rate ranging from about 0.5-2 volume of fresh medium/working volume of reactor/day (VVD) (e.g., about 0.5-1.5 VVD, about 0.75-1.5 VVD, about 0.75-1.25 VVD, about 1.0-2.0 VVD, about 1.0-1.9 VVD, about 1.0-1.8 VVD, about 1.0-1.7 VVD, about 1.0-1.6 VVD, about 1.0-1.5 VVD, about 1.0-1.4 VVD, about 1.0-1.3 VVD, about 1.0-1.2 VVD, about 1.0-1.1 VVD). In some embodiments, the perfusion process has a perfusion rate of about 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.05, 1.10, 1.15, 1.2, 1.25, 1.3, 1.35, 1.4, 1.45, 1.5, 1.55, 1.6, 1.65, 1.7, 1.75, 1.8, 1.85, 1.9, 1.95, or 2.0 VVD.

[0148] A perfusion process may also be characterized by volume of fresh medium added per cell per day, which is defined as cell specific perfusion rate. Various cell specific perfusion rates may be used. In some embodiments, the perfusion process has a cell specific perfusion rate ranging from about 0.05-5 nanoliter per cell per day (nL/cell/day) (e.g., about 0.05-4 nL/cell/day, about 0.05-3 nL/cell/day, about 0.05-2 nL/cell/day, about 0.05-1 nL/cell/day, about 0.1-5 nL/cell/day, about 0.1-4 nL/cell/day, about 0.1-3 nL/cell/day, about 0.1-2 nL/cell/day, about 0.1-1 nL/cell/day, about 0.15-5 nL/cell/day, about 0.15-4 nL/cell/day, about 0.15-3 nL/cell/day, about 0.15-2 nL/cell/day, about 0.15-1 nL/cell/day, about 0.2-5 nL/cell/day, about 0.2-4 nL/cell/day, about 0.2-3 nL/cell/day, about 0.2-2 nL/cell/day, about 0.2-1 nL/cell/day, about 0.25-5 nL/cell/day, about 0.25-4 nL/cell/day, about 0.25-3 nL/cell/day, about 0.25-2 nL/cell/day, about 0.25-1 nL/cell/day, about 0.3-5 nL/cell/day, about 0.3-4 nL/cell/day, about 0.3-3 nL/cell/day, about 0.3-2 nL/cell/day, about 0.3-1 nL/cell/day, about 0.35-5 nL/cell/day, about 0.35-4 nL/cell/day, about 0.35-3 nL/cell/day, about 0.35-2 nL/cell/day, about 0.35-1 nL/cell/day, about 0.4-5 nL/cell/day, about 0.4-4 nL/cell/day, about 0.4-3 nL/cell/day, about 0.4-2 nL/cell/day, about 0.4-1 nL/cell/day, about 0.45-5 nL/cell/day, about 0.45-4 nL/cell/day, about 0.45-3 nL/cell/day, about 0.45-2 nL/cell/day, about 0.45-1 nL/cell/day, about 0.5-5 nL/cell/day, about 0.5-4 nL/cell/day, about 0.5-3 nL/cell/day, about 0.5-2 nL/cell/day, about 0.5-1 nL/cell/day). In some embodiments, the perfusion process has a cell specific perfusion rate of about 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.1,

1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0 nL/cell/day.

[0149] The cell culture may be agitated or shaken during the production phase in order to increase oxygenation and dispersion of nutrients to the cells. In accordance with the present invention, one of ordinary skill in the art will understand that it can be beneficial to control or regulate certain internal conditions of the bioreactor during the growth phase, including but not limited to pH, temperature, oxygenation, etc. For example, pH can be controlled by supplying an appropriate amount of acid or base and oxygenation can be controlled with sparging devices that are well known in the art. One or more antiform agents may also be provided.

[0150] Same culture medium may be used throughout the production process including the growth phase, production phase and profusion. In some embodiments, at least two different media are used in the production of recombinant I2S. For example, a nutrient medium formulated for cell growth is often used to support growth of the cells throughout the cell growth phase, and nutrient medium formulated for protein production is used during the production phase of the process to support expression and harvesting of I2S. In either case, the nutrient medium may or may not contain serum or other animal-derived components (e.g., fetuin).

[0151] According to the present invention, the cells are typically grown in suspension. However, the cells may be attached to a substrate. In one example, cells may be attached to microbead or particles which are suspended in the nutrient medium.

Bioreactors

[0152] The invention also provides bioreactors that are useful for producing recombinant iduronate-2-sulfatase. Bioreactors may be perfusion, batch, fed-batch, repeated batch, or continuous (*e.g.* a continuous stirred-tank reactor models), for example. Typically, the bioreactors comprise at least one vessel designed and are configured to house medium (*e.g.*, a chemically defined nutrient medium). The vessel also typically comprises at least one inlet designed and configured to flow fresh nutrient medium into the vessel. The vessel also typically comprises at least one outlet designed and configured to flow waste medium out of the vessel. In some embodiments, the vessel may further comprise at least one filter designed

and configured to minimize the extent to which isolated cells in the vessel are passed out through the at least one outlet with waste medium. The bioreactor may also be fitted with one or more other components designed to maintain conditions suitable for cell growth. For example, the bioreactor may be fitted with one or more circulation or mixing devices designed and configured to circulate or mix the nutrient medium within the vessel. Typically, the isolated cells that are engineered to express recombinant I2S are suspended in the nutrient medium. Therefore, in some cases, the circulation device ensures that the isolated cells remain in suspension in the nutrient medium. In some cases, the cells are attached to a substrate. In some cases, the cells are attached to one or more substrates (*e.g.*, microbeads) that are suspended in the nutrient medium. The bioreactor may comprise one or more ports for obtaining a sample of the cell suspension from the vessel. The bioreactor may be configured with one or more components for monitoring and/or controlling conditions of the culture, including conditions such as gas content (*e.g.*, air, oxygen, nitrogen, carbon dioxide), flow rates, temperature, pH and dissolved oxygen levels, and agitation speed/circulation rate.

[0153] Vessels of any appropriate size may be used in the bioreactors. Typically, the vessel size is suitable for satisfying the production demands of manufacturing recombinant I2S. In some embodiments, the vessel is designed and configured to contain up to 1 L, up to 10 L, up to 100 L, up to 500 L, up to 1000 L, up to 1500 L, up to 2000 L, or more of the nutrient medium. In some embodiments, the volume of the production bioreactor is at least 10 L, at least 50 L, 100 L, at least 200 L, at least 250 L, at least 500 L, at least 1000 L, at least 1500 L, at least 2000 L, at least 2500 L, at least 5000 L, at least 8000 L, at least 10,000 L, or at least 12,000 L, or more, or any volume in between. The production bioreactor may be constructed of any material that is conducive to cell growth and viability that does not interfere with expression or stability or activity of the produced I2S protein. Exemplary material may include, but not be limited to, glass, plastic, or metal.

[0154] In some embodiments, cells may be cultured in a chemically defined medium that is housed in a vessel of a bioreactor. The culture methods often involve perfusing fresh nutrient medium into the vessel through the at least one inlet and bleeding waste nutrient medium out from vessel through the at least one outlet. Bleeding is performed at a rate of up to about 0.1 vessel volume per day, about 0.2 vessel volume per day, about 0.3 vessel volume per day, about 0.4 vessel volume per day, about 0.5 vessel volume per day, about 1 vessel volume per day, about 1.5 vessel volumes per day or more. The methods also involve

harvesting nutrient medium that comprises recombinant I2Ss. Harvesting may be performed at a rate of up to about 0.1 vessel volume per day, about 0.2 vessel volume per day, about 0.3 vessel volume per day, about 0.4 vessel volume per day, about 0.5 vessel volume per day, about 1 vessel volume per day, about 1.5 vessel volumes per day or more. Perfusing is also performed, typically at a rate equivalent to the sum of the bleeding rate and the harvesting rate. For example, perfusion rate may be great than about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0 vessel volume per day. In some embodiments, perfusion rate is less than about 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5 vessel volume per day. Exemplary perfusion rates are described throughout the specification.

Monitoring Culture Conditions

[0155] In certain embodiments of the present invention, the practitioner may find it beneficial or necessary to periodically monitor particular conditions of the growing cell culture. Monitoring cell culture conditions allows the practitioner to determine whether the cell culture is producing recombinant polypeptide or protein at suboptimal levels or whether the culture is about to enter into a suboptimal production phase. In order to monitor certain cell culture conditions, it will be necessary to remove small aliquots of the culture for analysis.

[0156] As non-limiting example, it may be beneficial or necessary to monitor temperature, pH, cell density, cell viability, integrated viable cell density, osmolarity, or titer or activity of the expressed I2S protein. Numerous techniques are well known in the art that will allow one of ordinary skill in the art to measure these conditions. For example, cell density may be measured using a hemacytometer, a Coulter counter, or Cell density examination (CEDEX). Viable cell density may be determined by staining a culture sample with Trypan blue. Since only dead cells take up the Trypan blue, viable cell density can be determined by counting the total number of cells, dividing the number of cells that take up the dye by the total number of cells, and taking the reciprocal. Alternatively, the level of the expressed I2S protein can be determined by standard molecular biology techniques such as coomassie staining of SDS-PAGE gels, Western blotting, Bradford assays, Lowry assays, Biuret assays, and UV absorbance. It may also be beneficial or necessary to monitor the

post-translational modifications of the expressed I2S prtoein, including phosphorylation and glycosylation.

Purification of Expressed I2S Protein

[0157] Various methods may be used to purify or isolate I2S protein produced according to various methods described herein. In some embodiments, the expressed I2S protein is secreted into the medium and thus cells and other solids may be removed, as by centrifugation or filtering for example, as a first step in the purification process. Alternatively or additionally, the expressed I2S protein is bound to the surface of the host cell. In this embodiment, the host cells (for example, yeast cells) expressing the polypeptide or protein are lysed for purification. Lysis of host cells (e.g., yeast cells) can be achieved by any number of means well known to those of ordinary skill in the art, including physical disruption by glass beads and exposure to high pH conditions.

[0158] The I2S protein may be isolated and purified by standard methods including, but not limited to, chromatography (e.g., ion exchange, affinity, size exclusion, and hydroxyapatite chromatography), gel filtration, centrifugation, or differential solubility, ethanol precipitation or by any other available technique for the purification of proteins (See, e.g., Scopes, Protein Purification Principles and Practice 2nd Edition, Springer-Verlag, New York, 1987; Higgins, S. J. and Hames, B. D. (eds.), Protein Expression: A Practical Approach, Oxford Univ Press, 1999; and Deutscher, M. P., Simon, M. I., Abelson, J. N. (eds.), Guide to Protein Purification: Methods in Enzymology (Methods in Enzymology Series, Vol 182), Academic Press, 1997, all incorporated herein by reference). For immunoaffinity chromatography in particular, the protein may be isolated by binding it to an affinity column comprising antibodies that were raised against that protein and were affixed to a stationary support. Alternatively, affinity tags such as an influenza coat sequence, poly-histidine, or glutathione-S-transferase can be attached to the protein by standard recombinant techniques to allow for easy purification by passage over the appropriate affinity column. Protease inhibitors such as phenyl methyl sulfonyl fluoride (PMSF), leupeptin, pepstatin or aprotinin may be added at any or all stages in order to reduce or eliminate degradation of the polypeptide or protein during the purification process. Protease inhibitors are particularly desired when cells must be lysed in order to isolate and purify the expressed polypeptide or protein.

[0159] Exemplary purification methods are described in the Examples sections below. Additional purification methods are described in the provisional application entitled "Purification of Recombinant I2S Protein" filed on herewith on even date, the entire disclosure of which is hereby incorporated by reference.

Pharmaceutical Composition and Administration

[0160] Purified recombinant I2S protein may be administered to a Hunter Syndrome patient in accordance with known methods. For example, purified recombinant I2S protein may be delivered intravenously, subcutaneously, intramuscularly, parenterally, transdermally, or transmucosally (*e.g.*, orally or nasally)).

[0161] In some embodiments, a recombinant I2S or a pharmaceutical composition containing the same is administered to a subject by intravenous administration.

[0162] In some embodiments, a recombinant I2S or a pharmaceutical composition containing the same is administered to a subject by intrathecal administration. As used herein, the term "intrathecal administration" or "intrathecal injection" refers to an injection into the spinal canal (intrathecal space surrounding the spinal cord). Various techniques may be used including, without limitation, lateral cerebroventricular injection through a burrhole or cisternal or lumbar puncture or the like. In some embodiments, "intrathecal administration" or "intrathecal delivery" according to the present invention refers to IT administration or delivery via the lumbar area or region, i.e., lumbar IT administration or delivery. As used herein, the term "lumbar region" or "lumbar area" refers to the area between the third and fourth lumbar (lower back) vertebrae and, more inclusively, the L2-S1 region of the spine.

[0163] In some embodiments, a recombinant I2S or a pharmaceutical composition containing the same is administered to the subject by subcutaneous (i.e., beneath the skin) administration. For such purposes, the formulation may be injected using a syringe. However, other devices for administration of the formulation are available such as injection devices (e.g., the Inject-ease™ and Genject™ devices); injector pens (such as the GenPen™); needleless devices (e.g., MediJector™ and BioJector™); and subcutaneous patch delivery systems.

[0164] In some embodiments, intrathecal administration may be used in conjunction with other routes of administration (e.g., intravenous, subcutaneously, intramuscularly, parenterally, transdermally, or transmucosally (*e.g.*, orally or nasally)).

[0165] The present invention contemplates single as well as multiple administrations of a therapeutically effective amount of a recombinant I2S or a pharmaceutical composition containing the same described herein. A recombinant I2S or a pharmaceutical composition containing the same can be administered at regular intervals, depending on the nature, severity and extent of the subject's condition (e.g., a lysosomal storage disease). In some embodiments, a therapeutically effective amount of a recombinant I2S or a pharmaceutical composition containing the same may be administered periodically at regular intervals (e.g., once every year, once every six months, once every five months, once every three months, bimonthly (once every two months), monthly (once every month), biweekly (once every two weeks), weekly, daily or continuously).

[0166] A recombinant I2S or a pharmaceutical composition containing the same can be formulated with a physiologically acceptable carrier or excipient to prepare a pharmaceutical composition. The carrier and therapeutic agent can be sterile. The formulation should suit the mode of administration.

[0167] Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (*e.g.*, NaCl), saline, buffered saline, alcohols, glycerol, ethanol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, sugars such as mannitol, sucrose, or others, dextrose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, *etc.*, as well as combinations thereof. The pharmaceutical preparations can, if desired, be mixed with auxiliary agents (*e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like) which do not deleteriously react with the active compounds or interference with their activity. In some embodiments, a water-soluble carrier suitable for intravenous administration is used.

[0168] The composition or medicament, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder.

The composition can also be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrollidone, sodium saccharine, cellulose, magnesium carbonate, *etc.*

[0169] The composition or medicament can be formulated in accordance with the routine procedures as a pharmaceutical composition adapted for administration to human beings. For example, in some embodiments, a composition for intravenous administration typically is a solution in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0170] As used herein, the term "therapeutically effective amount" is largely determined base on the total amount of the therapeutic agent contained in the pharmaceutical compositions of the present invention. Generally, a therapeutically effective amount is sufficient to achieve a meaningful benefit to the subject (e.g., treating, modulating, curing, preventing and/or ameliorating the underlying disease or condition). For example, a therapeutically effective amount may be an amount sufficient to achieve a desired therapeutic and/or prophylactic effect, such as an amount sufficient to modulate lysosomal enzyme receptors or their activity to thereby treat such lysosomal storage disease or the symptoms thereof (e.g., a reduction in or elimination of the presence or incidence of "zebra bodies" or cellular vacuolization following the administration of the compositions of the present invention to a subject). Generally, the amount of a therapeutic agent (e.g., a recombinant lysosomal enzyme) administered to a subject in need thereof will depend upon the characteristics of the subject. Such characteristics include the condition, disease severity, general health, age, sex and body weight of the subject. One of ordinary skill in the art will be readily able to determine appropriate dosages depending on these and other related factors.

In addition, both objective and subjective assays may optionally be employed to identify optimal dosage ranges.

[0171] A therapeutically effective amount is commonly administered in a dosing regimen that may comprise multiple unit doses. For any particular therapeutic protein, a therapeutically effective amount (and/or an appropriate unit dose within an effective dosing regimen) may vary, for example, depending on route of administration, on combination with other pharmaceutical agents. Also, the specific therapeutically effective amount (and/or unit dose) for any particular patient may depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific pharmaceutical agent employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and/or rate of excretion or metabolism of the specific fusion protein employed; the duration of the treatment; and like factors as is well known in the medical arts.

[0172] Additional exemplary pharmaceutical compositions and administration methods are described in PCT Publication WO2011/163649 entitled "Methods and Compositions for CNS Delivery of Iduronate-2-Sulfatase;" and provisional application serial no. 61/618,638 entitled "Subcutaneous administration of iduronate 2 sulfatase" filed on March 30, 2012, the entire disclosures of both of which are hereby incorporated by reference.

[0173] It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the enzyme replacement therapy and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed invention.

EXAMPLES

Example 1. Generation of Optimized Cell Line Co-expressing recombinant I2S and FGE

[0174] This example illustrates an exemplary cell line co-expressing recombinant I2S and FGE that can be used to produce recombinant I2S protein. It will be clear to one skilled in the art, that a number of alternative approaches, expression vectors and cloning techniques are available.

[0175] A typical mature form of human iduronate-2-sulfatase enzyme (I2S) is a 525-amino acid glycoprotein that undergoes extensive processing and post translational modification for enzyme activation, such as glycosylation and cysteine conversion to formylgycine (Figure 1). In mammalian cells, conserved cysteine residues within the I2S (i.e., at amino acid 59) enzyme are converted to formylglycine by the formylglycine generating enzyme (FGE). The conversion of cysteine to formylglycine within the active site of the I2S enzyme is an important step in generating the active form of the human sulfatase enzyme. The purpose of this experiment was to engineer an optimized human cell line co-expressing I2S and FEG for generating active recombinant I2S.

[0176] Figure 2 illustrates a number of exemplary construct designs for co-expression of I2S and FGE. For example, expression units of I2S and FGE can be located on separate vectors and the separate vectors can be co-transfected or transfected separately (Figure 2A). Alternatively, expression units of I2S and FGE can be located on the same vector (Figure 2B). In one configuration, I2S and FGE can be on the same vector but under the control of separate promoters, also referred to as separate cistrons (Figure 2B(1)). Alternatively, I2S and FGE can be designed as transcriptionally linked cistrons, that is, I2S and FGE are designed as one open reading frame under the control of a same promoter (Figure 2B(2)). Typically, an internal ribosome entry site (IRES) is designed to allow translation initiation in the middle of the messenger RNA (mRNA) (Figure 2B(2)).

[0177] A human cell line was engineered to co-express human I2S protein with the amino acid sequence shown in SEQ ID NO:2 and human formylglycine generating enzyme (FGE) with the amino acid sequence shown in SEQ ID NO:6.

SEQ ID NO: 2
> Full-lenth Precursor iduronate 2-sulfatase
MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDK
LVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAG
NFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCR
GPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPH
IPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPI
PVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEW
AKYSNFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVE
LVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNPREL
IAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHA
GELYFVDSDPLQDHNMYNDSQGGDLFQLLMP

SEQ ID NO:6
Full-length human FGE precursor:
MAAPALGLVCGRCPELGLVLLLLLLSLLCGAAGSQEAGTGAGAGSLAGSCGCGTPQ
RPGAHGSSAAAHRYSREANAPGPVPGERQLAHSKMVPIPAGVFTMGTDDPQIKQDG
EAPARRVTIDAFYMDAYEVSNTEFEKFVNSTGYLTEAEKFGDSFVFEGMLSEQVKTN
IQQAVAAAPWWLPVKGANWRHPEGPDSTILHRPDHPVLHVSWNDAVAYCTWAGK
RLPTEAEWEYSCRGGLHNRLFPWGNKLQPKGQHYANIWQGEFPVTNTGEDGFQGT
APVDAFPPNGYGLYNIVGNAWEWTSDWWTVHHSVEETLNPKGPPSGKDRVKKGGS
YMCHRSYCYRYRCAARSQNTPDSSASNLGFRCAADRLPTMD

[0178] Both I2S and FGE expression are controlled by a human CMV promoter. Translation of I2S mRNA results in synthesis of a 550 amino acid full length I2S protein (SEQ ID NO:2), which includes a 25 amino acid signal peptide. The signal peptide is removed and a soluble enzyme is secreted from the cell.

[0179] The bacterial neomycin phosphotransferase (neo) coding sequence and/or Blasticidin S Deaminase (BSD) gene were used to allow for selection of transfected cells using the neomycin analog G418 and/or blasticidin, respectively. In addition, the mouse dihydrofolate reductase (DHFR) gene was used on the I2S- and/or FGE-encoding vector(s) to allow for isolation of cell lines containing increased copies of the I2S- and/or FGE-encoding sequences by methotrexate (MTX) selection.

[0180] Cells producing I2S were isolated and subjected to appropriate drug selection to isolate cells with an increased number of copies of the transfected I2S and/or FGE genes. Quantification of I2S was performed by ELISA.

[0181] The cell population was also subjected to step-wise selection in methotrexate (MTX) to isolate cells with increased I2S productivity. I2S productivity was monitored during MTX selection by ELISA.

[0182] After several rounds of propagation, several I2S producing clones were then subjected to suspension adaptation in serum-free media through a stepwise reduction from DMEM containing 10% calf serum to serum free chemically defined media. Several individual clonal populations were established through limited dilution cloning. Colonies were screened by I2S enzyme activity assay and ELISA. Two stable cell lines 2D and 4D showed high percent viability and robust expression of I2S and were selected for further development.

Example 2. Serum-free Suspension Cell Culture

[0183] This example demonstrates that a serum-free cell culture system may be used to successfully cultivate a cell line co-expressing I2S and FGE to produce recombinant I2S.

Generating a Seed Culture

[0184] Briefly, a seed culture was established using the 2D or 4D cell lines of Example 1. Cells were transferred to a 250ml vented tissue culture shake flask containing serum-free chemically defined expansion medium, supplemented with Methotrexate for selection, adjusted with sodium bicarbonate to a pH of 7.3 and grown under standard conditions.

Cell Culture Expansion

[0185] Upon reaching the desired viable cell density, the initial seed culture was used to inoculate the first of a series of step-wise cell culture expansions consisting of a 500 ml tissue culture shake flask followed by 2x 1L tissue culture shake flasks. In each case, the preceding cell culture was transferred in its entirety to inoculate the subsequent larger culture flask, upon reaching a desired cell density.

[0186] A batch culture expansion was performed by transferring each of the 2x 1L cultures into a 10L Cellbag bioreactor® (Wave Europe), and adding expansion medium to a final weight of 2.5 kg. After reaching a desired cell density, new expansion medium was added to a final weight of 5.0 kg and the cells grown to a desired density. The 10L Cellbag was transferred to a Wave bioreactor® system (Wave Europe) and culture conditions were modified to allow for growth under continuous medium perfusion. Expansion growth medium was delivered at a target weight of 5.0 L per day (1.0 vvd) and samples were collected for off-line metabolite analysis of pH, glutamine, glutamate, glucose, ammonium, lactate, pCO_2 and osmolarity.

[0187] Upon reaching a desired cell density, the entire 10L cell culture was transferred to a 50L Wave Cellbag bioreactor®, containing 20 kg of fresh expansion medium, and again grown to a desired cell density.

Bioreactor Expansion

[0188] Cell expansion was next performed using a 200L disposable bioreactor and centrifuge perfusion device (Centritech® CELL II unit, Pneumatic Scale Corporation), which is designed to concentrate cells and clarify media for recycling during perfusion mediated cell culture. Expansion medium was inoculated with a portion of the 50L culture sufficient to achieve a desired cell density.

[0189] Next a portion of the 200L culture was used to seed a 2000L disposable bioreactor and centrifuge perfusion device (Centritech® CELL II unit, Pneumatic Scale Corporation). Cells were grown under batch growth conditions for two days. Following the two day growth, conditions were adjusted for continuous perfusion, initiating the start of the transition phase.

Bioreactor Production

[0190] For the production phase, two Centritech CELL II units were used. Production phase was started approximately 24 hours after the start of the transition phase, at which time the cells typically had achieved a desired cell density. Cell density was maintained for a desired production period, by regulating the bleed rate.

Example 3. Physiochemical and Biological Characterization of Recombinant I2S Enzyme Produced in Serum-free Cell Culture

[0191] The purpose of the example was to perform a detailed characterization of the recombinant I2S protein produced using the serum-free cell culture method described above.

SDS-PAGE

[0192] For this experiment, recombinant I2S protein was generated using the 2D and 4D human cell lines, in two separate serum-free cell culture reactions using the methods described above. Samples were collected during the Production Phase, and the purified I2S enzyme was analyzed by SDS-PAGE, and treated with silver stain for visualization. Figure 3 shows, that in each of the separate manufacturing experiments, I2S protein produced from the 2D and 4D cell lines under serum-free conditions migrated at the appropriate size (Lanes 5 and 6), as indicated upon comparison with the molecular weight protein standard (Lane 1) and commercially available I2S assay controls (Lanes 2 and 3). Furthermore, the

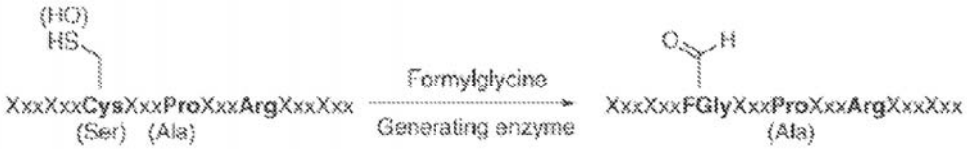
recombinant I2S produced under the serum-free condition (Lanes 5 and 6) also migrated at the same size as I2S Reference Standard (Lane 4).

Peptide Map

[0193] Recombinant I2S protein was generated using the I2S-AF 2D cell line grown under the serum-free culture conditions described above. The isolated recombinant I2S generated from the I2S-AF 2D cell line and a sample of reference human I2S were each subjected to proteolytic digest (e.g., by trypsin) and examined by HPLC analysis. Exemplary results are shown in Figure 4.

Percent Formylglycine Conversion

[0194] Peptide mapping can be used to determine Percent FGly conversion. I2S activation requires Cysteine (corresponding to position 59 of mature human I2S) to formylglycine conversion by formylglycine generating enzyme (FGE) as shown below:



Therefore, the percentage of formylglycine conversion (%FG) can be calculated using the following formula:

$$\%\mathbf{FG}\ (\text{of DS}) = \frac{\text{Number of active I2S molecules}}{\text{Number of total (active+inactive) I2S molecules}} \times 100$$

[0195] For example 50% FG means half of the purified recombinant I2S is enzymatically inactive without any therapeutic effect.

[0196] Peptide mapping was used to calculate %FG. Briefly, a recombinant I2S protein was digested into short peptides using a protease (e.g., trypsin or chymotrypsin). Short peptides were separated and characterized using HPLC. The peptide containing the position corresponding to position 59 of the mature human I2S was characterized to

determine if the Cys at position 59 was converted to a FGly as compared to a control (e.g., an I2S protein without FGly conversion or an I2S protein with 100% FGly conversion). The amount of peptides containing FGly (corresponding to number of active I2S molecules) and the total amount of peptides with both FGly and Cys (corresponding to number of total I2S molecules) may be determined based on the corresponding peak areas and the ratio reflecting %FG was calculated. Exemplary results are shown in Table 4.

Glycan Map – Mannose-6-Phosphate and Sialic Acid Content

[0197] The glycan and sialic acid composition of recombinant I2S protein produced under serum-free cell culture conditions was determined. Quantification of the glycan composition was performed, using anion exchange chromatography. As described below, the glycan map of recombinant I2S generated under these conditions consists of seven peak groups, eluting according to an increasing amount of negative charges, at least partly derived from sialic acid and mannose-6-phosphate glycoforms resulting from enzymatic digest. Briefly, purified recombinant I2S obtained using the serum-free cell culture method (I2S-AF 2D Serum-free and I2S-AF 4D Serum-free) and reference recombinant I2S produced, were treated with either (1) purified neuraminidase enzyme (isolated from Arthrobacter Ureafaciens (10 mU/μL), Roche Biochemical (Indianapolis, IN), Cat. # 269 611 (1U/100 μL)) for the removal of sialic acid residues, (2) alkaline phosphatase for 2 hours at 37±1°C for complete release of mannose-6-phosphate residues, (3) alkaline phosphatase + neuraminidase, or (4) no treatment. Each enzymatic digest was analyzed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) using a CarboPac PA1 Analytical Column equipped with a Dionex CarboPac PA1 Guard Column. A series of sialic acid and mannose-6-phosphate standards in the range of 0.4 to 2.0 nmoles were run for each assay. An isocratic method using 48 mM sodium acetate in 100 mM sodium hydroxide was run for a minimum of 15 minutes at a flow rate of 1.0 mL/min at ambient column temperature to elute each peak. The data generated from each individual run, for both the I2S-AF and reference I2S samples, were each combined into a single chromatograph to represent the glycan map for each respective recombinant protein. As indicated in Figure 5, an exemplary glycan map for I2S produced using the human cell serum-free method displayed representative elution peaks (in the order of elution) constituting neutrals, mono-, disialyated, monophosphorylated, trisialyated and hybrid

(monosialyated and capped mannose-6-phosphate), tetrasialylated and hybrid (disilaylated and capped mannose-6-phosphate) and diphosphorylated glycans.

[0198] Average sialic acid content (moles sialic acid per mole protein) in each recombinant I2S sample was calculated from linear regression analysis of sialic acid standards. Each chromatogram run was visualize using the PeakNet 6 Software. Sialic acid standards and sialic acid released from recombinant I2S assay control and test samples appear as a single peak. The amount of sialic acid (nmoles) for I2S was calculated as a raw value using the following equation:

$$S.A.(mole\ per\ mole\ I2S) = \frac{(nmoles\ sialic\ acid)}{(0.3272)(C)}$$

Where C is the protein concentration (in mg/ml) of sample or recombinant I2S assay control.

The corrected value of sialic acid as moles of sialic acid per mole of protein for each test sample was calculated using the following formula:

$$Corrected\ S.A. = \frac{(Sample\ Raw\ Sialic\ Acid\ Value)x(Established\ Idursulfase\ Assay\ Control\ Value)}{(Idursulfase\ Assay\ Control\ Raw\ Sialic\ Acid\ Value)}$$

[0199] Exemplary data indicative of sialic acid content on the recombinant I2S produced by I2S-AF 2D or 4D cell lines are shown in Table 4.

Table 4: Exemplary Characteristics of Recombinant I2S Produced in Serum-Free Cell Culture

Assay	I2S-AF 2D (Serum-free)
Peptide Mapping	
L1	101
L10	100
L12	102
L13	97
L14	101
L17	100
L20	102
Host Cell Protein	< 62.5 ng/mg
Ion Exchange HPLC % Area	
Peak A	62
Peak A+B	82
Peak E+F	0
% Formylglycine	87
Specific activity (U/mg) (sulfate release assay)	64
% Size Exclusion HPLC	≥ 99.8
Glycan Mapping	
Monosialylated	105
Disialylated	93
Monophosphorylated	139
Trisialylated	89
Tetrasialylated	125
Diphosphorylated	95
Sialic Acid (mol/mol)	20

Specific Activity

[0200] Specific activity of the recombinant I2S enzyme produced using the 2D and 4D cell lines under serum-free cell culture conditions was analyzed using *in vitro* sulfate release assay or 4-MUF assay.

In vitro sulfate release assay

[0201] *In vitro* sulfate release activity assay was conducted using heparin disaccharide as substrate. In particular, this assay measures the ability of I2S to release sulfate ions from a naturally derived substrate, heparin diasaccharide. The released sulfate may be quantified by ion chromatography equipped with a conductivity detector. Briefly, samples were first buffer exchanged to 10 mM Na acetate, pH 6 to remove inhibition by

phosphate ions in the formulation buffer. Samples were then diluted to 0.075 mg/ml with reaction buffer (10 mM Na acetate, pH 4.4) and incubated for 2 hrs at 37°C with heparin disaccharide at an enzyme to substrate ratio of 0.3 μg I2S/100 μg substrate in a 30 μL reaction volume. The reaction was then stopped by heating the samples at 100°C for 3 min. The analysis was carried out using a Dionex IonPac AS18 analytical column with an IonPac AG18 guard column. An isocratic method was used with 30 mM potassium hydroxide at 1.0 mL/min for 15 minutes. The amount of sulfate released by the I2S sample was calculated from the linear regression analysis of sulfate standards in the range of 1.7 to 16.0 nmoles. The reportable value was expressed as Units per mg protein, where 1 unit is defined as 1 μmoles of sulfate released per hour and the protein concentration is determined by A280 measurements. Exemplary results are shown in Table 4.

4-MUF assay

[0202] Specific activity of the recombinant I2S enzyme produced using the 2D and 4D cell lines under serum-free cell culture conditions may also be analyzed using the fluorescence based 4-MUF assay. Briefly, the assay measures the hydrolysis of I2S substrate 4-methylumbelliferyl-sulfate (4-MUF-SO_4). Upon cleavage of the 4-MUF-SO_4 substrate by I2S, the molecule is converted to sulfate and naturally fluorescent 4-methylumbelliferone (4-MUF). As a result, I2S enzyme activity can be determined by evaluating the overall change in fluorescent signal over time. For this experiment, purified I2S enzyme produced from the I2S-AF 2D and 4D human cell lines were incubated with a solution of 4-methylumbelliferyl-sulfate (4-MUF-SO_4), Potassium Salt, Sigma Cat. # M-7133). Calibration of the assay was performed using a series of control reference samples, using commercially available I2S enzyme diluted at 1:100, 1:200 and 1:20,000 of the stock solution. The enzymatic assay was run at 37°C and assayed using a calibrated fluorometer. Using the fluorescence values obtained for each reference standard, the percent coefficient of variation was determined using the following equation:

$$\%CV = \frac{S\tan dard\ Deviation of\ Raw Fluorescenc Values(N=3)}{Averagel\ Fluorescence Value} X\,100\%$$

[0203] The percent CV values were then used to calculate the Corrected Average Fluorescence for each sample, in order to determine the reportable enzyme activity, expressed in mU/mL using the following formula:

$$mU/mL = (CFU)\left(\frac{1\,nmole/L}{10\,FU}\right)\left(\frac{1L}{10^3\,mL}\right)\left(\frac{2.11\,mL}{0.01\,mL}\right)\left(\frac{1\,hour}{60\,\min}\right)\left(\frac{1\,mU}{nmole}\right)(DF)$$

CFU = Negative corrected average fluorescence

DF - Dilution Factor

[0204] One milliunit of activity is the quantity of enzyme required to convert 1 nanomole of 4-methylumbelliferyl-sulfate to 4-methylumbelliferone in 1 minute at 37°C.

Charge Profile

[0205] The charge distribution of each purified recombinant I2S was determined by Strong Anion Exchange (SAX) Chromatography, with a High Performance Liquid Chromatography (HPLC) system. The method separates recombinant I2S variants within the sample, based on surface charge differences. At pH 8.00, negatively charged species adsorb onto the fixed positive charge of the SAX column. A gradient of increasing ionic strength is used to elute each protein species in proportion to the strength of their ionic interaction with the column. One hundred micrograms of purified I2S, isolated from the 2D cell line under serum-free growth conditions or reference recombinant I2S enzyme, was loaded onto an Amersham Biosciences Mini Q PE (4.6 x 50 mm) column held at ambient temperature and equilibrated to 20 mM Tris-HC1, pH 8.00. Gradient elution was made at a flow rate of 0.80 mL/min, using a mobile phase of 20 mM Tris-HC1, 1.0 M sodium chloride, pH 8.00. Protein concentration was continuously determined during the run, by measuring light absorbance of the sample elution at the 280 nm wavelength. Exemplary results are shown in Figure 6.

[0206] While certain compounds, compositions and methods described herein have been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the compounds of the invention and are not intended to limit the same.

[0207] The articles "a" and "an" as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in,

employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or the entire group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Where elements are presented as lists, (e.g., in Markush group or similar format) it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in so many words herein. It should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. The publications, websites and other reference materials referenced herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference.

We claim:

1. A method for large-scale production of recombinant iduronate-2-sulfatase (I2S) protein in mammalian cells, comprising culturing mammalian cells co-expressing a recombinant I2S protein and a formylglycine generating enzyme (FGE) in suspension in a large-scale culture vessel containing medium lacking serum.

2. A method for large-scale production of recombinant iduronate-2-sulfatase (I2S) protein in mammalian cells, comprising culturing mammalian cells co-expressing a recombinant I2S protein and a formylglycine generating enzyme (FGE) in a large-scale culture vessel containing medium lacking serum under conditions such that the cells, on average, produce the recombinant I2S protein at a specific productivity rate of greater than about 15 picogram/cell/day and further wherein the produced recombinant I2S protein, on average, comprises at least about 60% conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_α-formylglycine.

3. The method of claim 1 or 2, wherein the culturing step comprises a perfusion process.

4. The method of claim 3, wherein the perfusion process has a perfusion rate ranging from about 0.5-2 volume of fresh medium/working volume of reactor/day (VVD).

5. The method of claim 3, wherein the perfusion process has a cell specific perfusion rate ranging from about 0.05-5 nanoliter per cell per day (nL/cell/day).

6. The method of any one of the preceding claims, wherein the cells, on average, produce the recombinant I2S protein at a specific productivity rate of great than about 30 picogram/cell/day.

7. The method of any one of the preceding claims, wherein the cells produce the recombinant I2S protein at an average harvest titer of at least 6 mg per liter per day.

8. The method of any one of the preceding claims, wherein the produced recombinant I2S protein, on average, comprises at least about 70% conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_α-formylglycine.

9. The method of any one of the preceding claims, wherein the produced recombinant I2S protein comprises at least about 80% conversion of the cysteine residue corresponding to Cys59 of human I2S protein to FGly.

10. The method of any one of the preceding claims, wherein the produced recombinant I2S protein comprises at least about 97% conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_α-formylglycine (FGly).

11. The method of any one of the preceding claims, wherein the mammalian cells are human cells.

12. The method of any one of claims 1-10, wherein the mammalian cells are CHO cells.

13. The method of any one of the preceding claims, wherein the large-scale culture vessel is a bioreactor.

14. The method of claim 13, wherein the bioreactor is at a scale of or greater than 10L, 200L, 500L, 1000L, 1500L, or 2000L.

15. The method of any one of the preceding claims, wherein the medium lacks animal-derived components.

16. The method of any one of the preceding claims, wherein the medium is chemically-defined medium.

17. The method of any one of the preceding claims, wherein the medium is protein free.

18. The method of any one of the preceding claims, wherein the medium comprises at least one redox-modulator.

19. The method of claim 18, wherein the at least one redox-modulator is selected from the group consisting of glutathione, glucose-6-phosphate, carnosine, carnosol, sulforaphane, tocopherol, ascorbate, dehydroascorbate, selenium, 2-mercaptoenthanol, N-acetylcysteine,

cysteine, riboflavin, niacin, folate, flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADP).

20. The method of claim 19, wherein the at least one redox-modulator comprises cysteine.

21. The method of claim 20, wherein the cysteine is at a concentration ranging from about 0.1 mg/L to about 65 mg/L.

22. The method of any one of claims 18-21, wherein the at least one redox-modulator comprises 2-mercaptoenthanol.

23. The method of claim 22, wherein the 2-mercaptoenthanol is at a concentration ranging from about 0.001 mM to about 0.01 mM.

24. The method of any one of claims 18-23, wherein the at least one redox-modulator comprises N-acetylcysteine.

25. The method of claim 24, wherein the N-acetylcysteine is at a concentration ranging from about 3 mM to about 9 mM.

26. The method of any one of the preceding claims, wherein the medium comprises at least one growth-modulator.

27. The method of claim 26, wherein the at least one growth-modulator comprises hypoxanthine.

28. The method of claim 27, wherein the hypoxanthine is at a concentration ranging from about 0.1 mM to about 10 mM.

29. The method of any one of claims 26-28, wherein the at least one growth-modulator comprises thymidine.

30. The method of claim 29, wherein the thymidine is at a concentration ranging from about 1 mM to about 100 mM.

31. The method of any one of the preceding claims, wherein the medium has a pH ranging from about 6.8 – 7.5.

32. The method of claim 31, wherein the medium has a pH ranging from about 6.9-7.3.

33. The method of any one of the preceding claims, wherein the culturing step comprises a growth phase and a production phase.

34. The method of any one of the preceding claims, wherein the mammalian cells are cultured at a temperature ranging from 30-37 °C.

35. The method of claim 33 or 34, wherein the mammalian cells are cultured at different temperatures during the growth phase and the production phase.

36. The method of any one of claims 33-35, wherein the medium for the growth phase and the production phase has different pH.

37. The method of any one of claims 33-36, wherein the mammalian cells are maintained at a viable cell density ranging from about 1.0-50 X 10^6 viable cells/mL during the production phase.

38. The method of any one of claims 33-37, wherein the production phase is lasted for about 5-90 days.

39. The method of any one of the preceding claims, wherein the method further comprises a step of harvesting the recombinant I2S protein.

40. The method of any one of the preceding claims, wherein the recombinant I2S protein comprises an amino acid sequence at least 70% identical to SEQ ID NO:1.

41. The method of claim 40, wherein the recombinant I2S protein comprises an amino acid sequence identical to SEQ ID NO:1.

42. The method of any one of the preceding claims, wherein the FGE comprises an amino acid sequence at least 70% identical to SEQ ID NO:5.

43. The method of claim 42, wherein the FGE comprises an amino acid sequence identical to SEQ ID NO:5.

44. The method of any one of the preceding claims, wherein the cells comprises one or more exogenous nucleic acids encoding the recombinant I2S protein and/or the FGE.

45. The method of claim 44, wherein the one or more exogenous nucleic acids are integrated in the genome of the cells.

46. The method of claim 44, wherein the one or more exogenous nucleic acids are present on one or more extra-chromosomal constructs.

47. The method of any one the preceding claims, wherein the cells over-express the recombinant I2S protein.

48. The method of any one the preceding claims, wherein the cells over-express the FGE.

49. A recombinant iduronate-2-sulfatase (I2S) protein produced using a method of any one of the preceding claims.

50. A preparation of recombinant iduronate-2-sulfatase (I2S) protein, said recombinant I2S protein having an amino acid sequence at least 70% identical to SEQ ID NO:1 and comprising at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to C_{α}-formylglycine (FGly).

51. The preparation of claim 50, wherein the recombinant I2S protein comprises at least about 80% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to FGly.

52. The preparation of claim 50 or 51, wherein the recombinant I2S protein comprises at least about 90% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to FGly.

53. The preparation of any one of claims 50-52, wherein the recombinant I2S protein comprises at least about 95% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to FGly.

54. The preparation of any one of claims 50-53, wherein the recombinant I2S protein comprises at least about 97% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to FGly.

55. The preparation of any one of claims 50-54, wherein the recombinant I2S protein has specific activity of at least 40 U/mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

56. The preparation of any one of claims 50-55, wherein the recombinant I2S protein has specific activity of at least 60 U/mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

57. The preparation of any one of claims 50-56, wherein the recombinant I2S protein has specific activity of at least 80 U/mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

58. The preparation of any one of claims 50-57, wherein the recombinant I2S protein comprises an amino acid sequence at least 70% identical to SEQ ID NO:1.

59. The preparation of any one of claims 50-58, wherein the recombinant I2S protein comprises an amino acid sequence identical to SEQ ID NO:1.

60. A pharmaceutical composition comprising a recombinant I2S protein of any one of claims 50-59 and a pharmaceutically acceptable carrier.

61. A method of treating Hunter syndrome comprising administering into a subject in need of treatment a pharmaceutical composition of claim 60.

SEQ ID NO: 1

```
1   Ser Glu Thr Gln Ala [Asn] Ser Thr Thr Asp Ala Leu Asn Val Leu Leu Ile Ile Val Asp
21  Asp Leu Arg Pro Ser Leu Gly Cys Tyr Gly Asp Lys Leu Val Arg Ser Pro Asn Ile Asp
41  Gln Leu Ala Ser His Ser Leu Leu Phe Gln Asn Ala Phe Ala Gln Gln Ala Val [Cys] Ala
61  Pro Ser Arg Val Ser Phe Leu Thr Gly Arg Arg Pro Asp Thr Thr Arg Leu Tyr Asp Phe
81  Asn Ser Tyr Trp Arg Val His Ala Gly [Asn] Phe Ser Thr Ile Pro Gln Tyr Phe Lys Glu
101 Asn Gly Cys Val Thr Met Ser Val Gly Lys Val Phe His Pro Gly Ile Ser Ser [Asn] Ala
121 Thr Asp Asp Ser Pro Tyr Ser Trp Ser Phe Pro Pro Tyr His Pro Ser Ser Glu Lys Tyr
141 Glu Asn Thr Lys Thr Cys Arg Gly Pro Asp Gly Glu Leu His Ala Asn Leu Leu Cys Pro
161 Val Asp Val Leu Asp Val Pro Glu Gly Thr Leu Pro Asp Lys Gln Ser Thr Glu Gln Ala
181 Ile Gln Leu Leu Glu Lys Met Lys Thr Ser Ala Ser Pro Phe Phe Leu Ala Val Gly Tyr
201 His Lys Pro His Ile Pro Phe Arg Tyr Pro Lys Glu Phe Gln Lys Leu Tyr Pro Leu Glu
221 [Asn] Ile Thr Leu Ala Pro Asp Pro Glu Val Pro Asp Gly Leu Pro Pro Val Ala Tyr Asn
241 Pro Trp Met Asp Ile Arg Gln Arg Glu Asp Val Gln Ala Leu [Asn] Ile Ser Val Pro Tyr
261 Gly Pro Ile Pro Val Asp Phe Gln Arg Lys Ile Arg Gln Ser Tyr Phe Ala Ser Val Ser
281 Tyr Leu Asp Thr Gln Val Gly Arg Leu Leu Ser Ala Leu Asp Asp Leu Gln Leu Ala [Asn]
301 Ser Thr Ile Ile Ala Phe Thr Ser Asp His Gly Trp Ala Leu Gly Glu His Gly Glu Trp
321 Ala Lys Tyr Ser Asn Phe Asp Val Ala Thr His Val Pro Leu Ile Phe Tyr Val Pro Gly
341 Arg Thr Ala Ser Leu Pro Glu Ala Gly Glu Lys Leu Phe Pro Tyr Leu Asp Pro Phe Asp
361 Ser Ala Ser Gln Leu Met Glu Pro Gly Arg Gln Ser Met Asp Leu Val Glu Leu Val Ser
381 Leu Phe Pro Thr Leu Ala Gly Leu Ala Gly Leu Gln Val Pro Pro Arg Cys Pro Val Pro
401 Ser Phe His Val Glu Leu Cys Arg Glu Gly Lys Asn Leu Leu Lys His Phe Arg Phe Arg
421 Asp Leu Glu Glu Asp Pro Tyr Leu Pro Gly Asn Pro Arg Glu Leu Ile Ala Tyr Ser Gln
441 Tyr Pro Arg Pro Ser Asp Ile Pro Gln Trp Asn Ser Asp Lys Pro Ser Leu Lys Asp Ile
461 Lys Ile Met Gly Tyr Ser Ile Arg Thr Ile Asp Tyr Arg Tyr Thr Val Trp Val Gly Phe
481 Asn Pro Asp Glu Phe Leu Ala [Asn] Phe Ser Asp Ile His Ala Gly Glu Leu Tyr Phe Val
501 Asp Ser Asp Pro Leu Gln Asp His Asn Met Tyr [Asn] Asp Ser Gln Gly Gly Asp Leu Phe
521 Gln Leu Leu Met Pro
```

[Asn] - marks sites of N-linked glycosylation

[Cys] - example site of cysteine conversion

Fig. 1

I2S and SUMF1 co-expression options

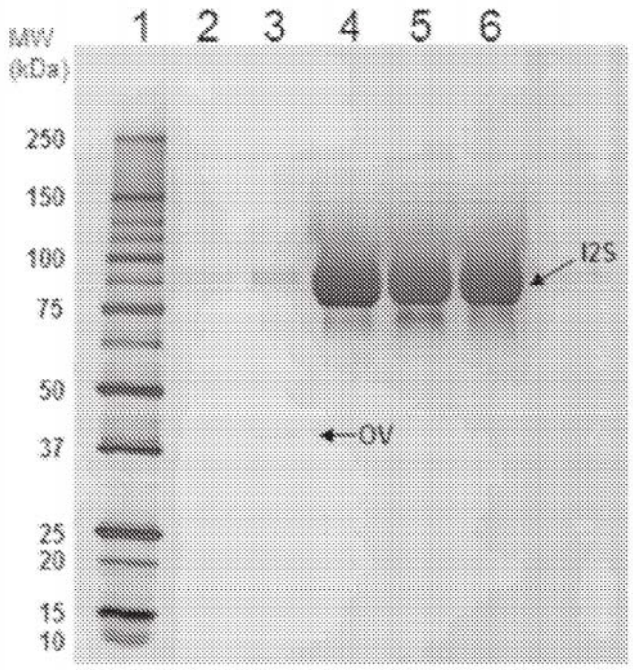
A) Expression units on separate vectors (co-transfection or subsequent transfections)

B) Expression units on the same vector (one transfection)

1) Separate cistrons

2) Transcriptionally linked cistrons

Fig. 2



Lane	Load Sample	Load Volume	Total µg
1	Protein Stds	3 µl	
2	Assay Control #1	20 µl	8 µg
3	Assay Control #2	20 µl	16 µg
4	I2S Reference Standard	20 µl	8 µg
5	I2S-AF 2D Serum-free Culture	20 µl	8 µg
6	I2S-AF 4D Serum-free Culture	20 µl	8 µg

Fig. 3

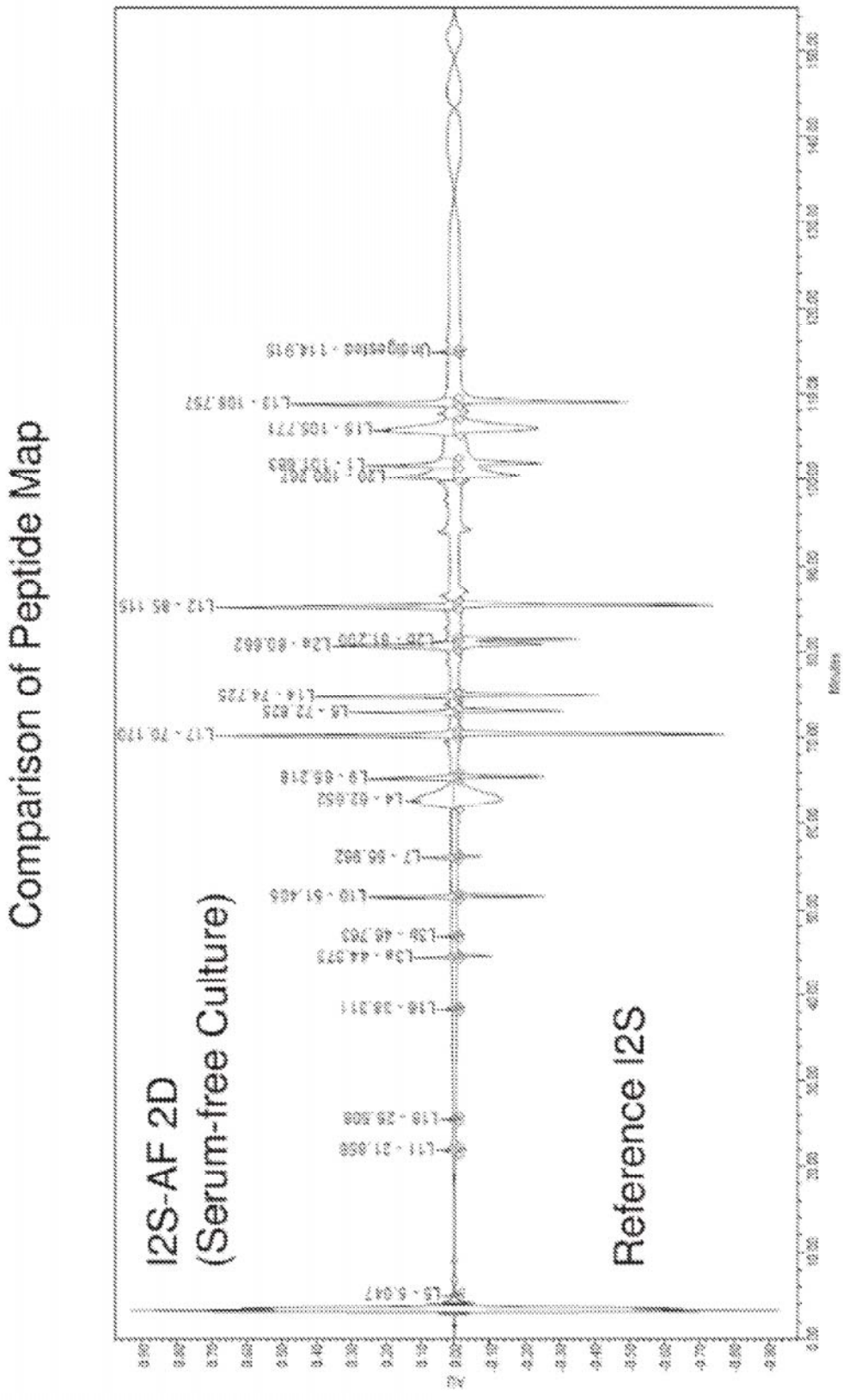

Comparison of Peptide Map
I2S-AF 2D
(Serum-free Culture)
Reference I2S
Minutes

Fig. 4

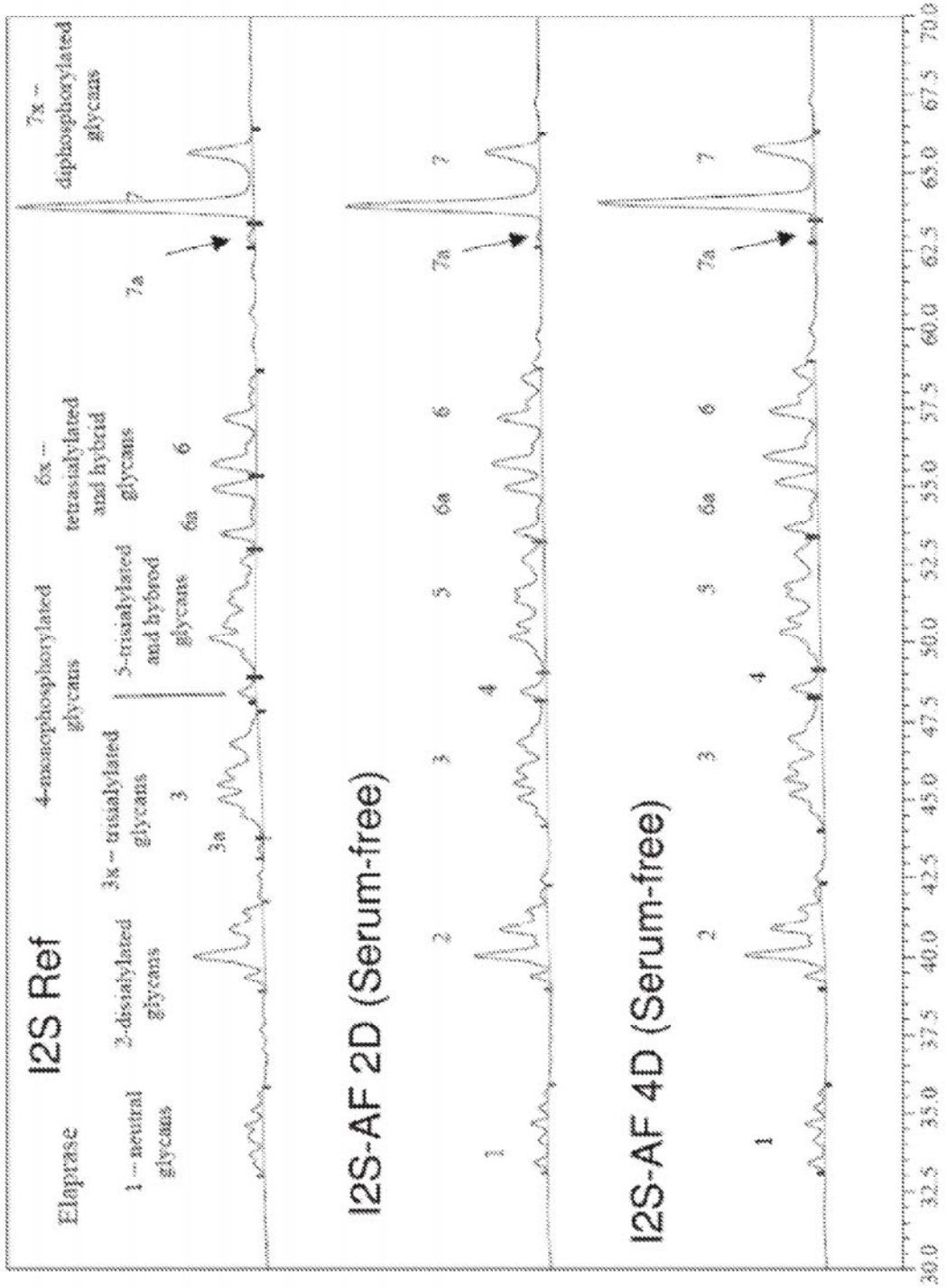

I2S Ref
Elaprase
1 – neutral glycans
2-disialylated glycans
3x – trisialylated glycans
3
3a
4-monophosphorylated glycans
3-trisialylated and hybrid glycans
6a
6x – tetrasialylated and hybrid glycans
6
7a
7x – diphosphorylated glycans
7
I2S-AF 2D (Serum-free)
1
2
3
4
5
6a
6
7a
7
I2S-AF 4D (Serum-free)
1
2
3
4
5
6a
6
7a
7
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
70.0

Fig. 5

I2S Derived Using I2S-AF 2D Serum-free Cell Culture

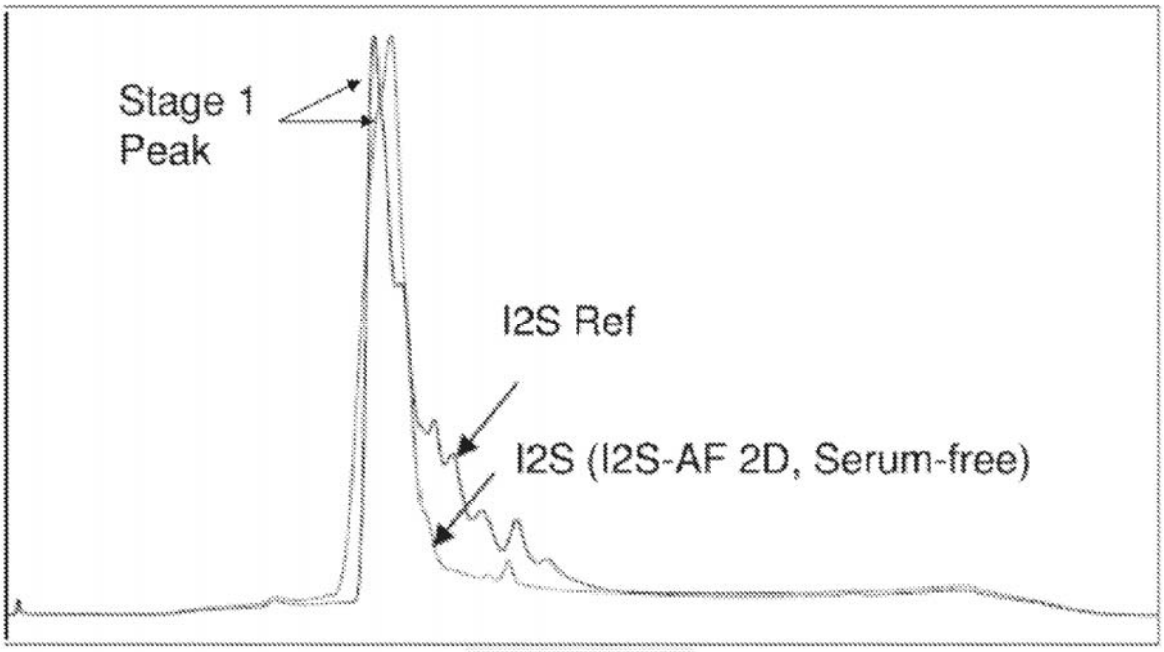


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/48601

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 21/02 (2013.1)
USPC - 435/68.1, 69.1, 41; 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)
IPC(8): C12P 21/06 (2013.1)
USPC: 435/68.1, 41; 530/350

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; Pubmed; Science Direct; 'Iduronate-2-sulfatase,' 'I2S,' 'lysosomal sulfatase,' recombinant, 'picogram/cell/day'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 8128925 B2 (VELLARD, MC et al.) March 6, 2012; column 2, lines 55-60; column 25, lines 47-57; column 45, lines 1-4	1, 3/1, 5/3/1 ----- 2, 3/2, 4/3/1, 4/3/2, 5/3/2, 50, 51, 52/50, 52/51
Y	WO 2005/113765 A2 (ZANKEL, T et al.) December 1, 2005; page 25, lines 5-11; page 25, lines 26-33 to page 26, lines 1-8	2, 3/2, 4/3/2, 5/3/2
Y	WO 2011/044542 A1 (PARDRIDGE, WM et al.) April 14, 2011; paragraphs [0003], [0099], [00161], [00183]; figure 4	2, 3/2, 5/3/2, 50, 51, 52/50, 52/51
Y	US 7691611 B2 (WEBER, U et al.) April 6, 2010; Claims 1-3	4/3/1, 4/3/2

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 2013 (25.11.2013)	03 DEC 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/48601

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/48601

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ 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:

3. ☒ Claims Nos.: 6-49, 53-61
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ 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.:

4. ☐ 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.:

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.

(19)中华人民共和国国家知识产权局

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(51)Int.Cl.
C12P 21/02(2006.01)

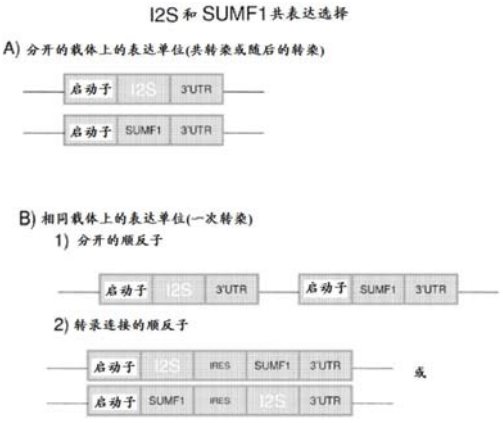
权利要求书3页 说明书37页
序列表13页 附图6页

(54)发明名称

产生重组艾杜糖-2-硫酸酯酶的方法

(57)摘要

本发明除其它事项以外提供了在无血清培养基中使用哺乳动物细胞的悬浮培养大规模产生重组I2S蛋白的方法和组合物。具体地,本发明使用共表达重组I2S蛋白和甲酰甘氨酸生成酶(FGE)的哺乳动物细胞。



CN 104583414 A

1. 一种用于在哺乳动物细胞中大规模产生重组艾杜糖 2- 硫酸酯酶 (I2S) 蛋白的方法，所述方法包括在含有缺乏血清的培养基的大规模培养皿中悬浮培养共表达重组 I2S 蛋白和甲酰甘氨酸生成酶 (FGE) 的哺乳动物细胞。

2. 一种用于在哺乳动物细胞中大规模产生重组艾杜糖 2- 硫酸酯酶 (I2S) 蛋白的方法，所述方法包括在一定条件下，在含有缺乏血清的培养基的大规模培养皿中培养共表达重组 I2S 蛋白和甲酰甘氨酸生成酶 (FGE) 的哺乳动物细胞，以便所述细胞平均以大于约 15 皮克 / 细胞 / 日的比生产率产生所述重组 I2S 蛋白，并且另外地其中所述产生的重组 I2S 蛋白平均包含至少约 60％的对应于人 I2S 蛋白的 Cys59 的所述半胱氨酸残基至 C_{α}- 甲酰甘氨酸的转化。

3. 根据权利要求 1 或 2 所述的方法，其中所述培养步骤包括灌注工艺。

4. 根据权利要求 3 所述的方法，其中所述灌注工艺具有在约 0.5-2 体积的新鲜培养基 / 反应器的工作体积 / 日 (VVD) 的范围内的灌注速率。

5. 根据权利要求 3 所述的方法，其中所述灌注工艺具有在约 0.05-5 纳升每种细胞每日 (nL/ 细胞 / 日) 的范围内的细胞比灌注速率。

6. 根据前述权利要求中任一项所述的方法，其中所述细胞平均以大于约 30 皮克 / 细胞 / 日的比生产率产生所述重组 I2S 蛋白。

7. 根据前述权利要求中任一项所述的方法，其中所述细胞以至少 6mg 每升每日的平均收获滴度产生所述重组 I2S 蛋白。

8. 根据前述权利要求中任一项所述的方法，其中所述产生的重组 I2S 蛋白平均包含至少约 70％的对应于人 I2S 蛋白的 Cys59 的所述半胱氨酸残基至 C_{α}- 甲酰甘氨酸的转化。

9. 根据前述权利要求中任一项所述的方法，其中所述产生的重组 I2S 蛋白包含至少约 80％的对应于人 I2S 蛋白的 Cys59 的所述半胱氨酸残基至 FGly 的转化。

10. 根据前述权利要求中任一项所述的方法，其中所述产生的重组 I2S 蛋白包含至少约 97％的对应于人 I2S 蛋白的 Cys59 的所述半胱氨酸残基至 C_{α}- 甲酰甘氨酸 (FGly) 的转化。

11. 根据前述权利要求中任一项所述的方法，其中所述哺乳动物细胞为人细胞。

12. 根据权利要求 1-10 中任一项所述的方法，其中所述哺乳动物细胞为 CHO 细胞。

13. 根据前述权利要求中任一项所述的方法，其中所述大规模培养皿为生物反应器。

14. 根据权利要求 13 所述的方法，其中所述生物反应器为或大于 10L、200L、500L、1000L、1500L 或 2000L 的规模。

15. 根据前述权利要求中任一项所述的方法，其中所述培养基缺乏动物来源的组分。

16. 根据前述权利要求中任一项所述的方法，其中所述培养基为化学成分确定的培养基。

17. 根据前述权利要求中任一项所述的方法，其中所述培养基不含蛋白质。

18. 根据前述权利要求中任一项所述的方法，其中所述培养基包含至少一种氧化还原调节剂。

19. 根据权利要求 18 所述的方法，其中所述至少一种氧化还原调节剂选自谷胱甘肽、葡萄糖 -6- 磷酸、肌肽、鼠尾草酚、萝卜硫素、生育酚、抗坏血酸、脱氢抗坏血酸、硒、2- 巯基乙醇、N- 乙酰半胱氨酸、半胱氨酸、核黄素、烟酸、叶酸、黄素腺嘌呤二核苷酸 (FAD) 和烟酰

胺腺嘌呤二核苷酸磷酸 (NADP)。

20. 根据权利要求 19 所述的方法，其中所述至少一种氧化还原调节剂包括半胱氨酸。

21. 根据权利要求 20 所述的方法，其中所述半胱氨酸的浓度在约 0.1mg/L 至约 65mg/L 的范围内。

22. 根据权利要求 18-21 中任一项所述的方法，其中所述至少一种氧化还原调节剂包括 2- 巯基乙醇。

23. 根据权利要求 22 所述的方法，其中所述 2- 巯基乙醇的浓度在约 0.001mM 至约 0.01mM 的范围内。

24. 根据权利要求 18-23 中任一项所述的方法，其中所述至少一种氧化还原调节剂包括 N- 乙酰半胱氨酸。

25. 根据权利要求 24 所述的方法，其中所述 N- 乙酰半胱氨酸的浓度在约 3mM 至约 9mM 的范围内。

26. 根据前述权利要求中任一项所述的方法，其中所述培养基包含至少一种生长调节剂。

27. 根据权利要求 26 所述的方法，其中所述至少一种生长调节剂包括次黄嘌呤。

28. 根据权利要求 27 所述的方法，其中所述次黄嘌呤的浓度在约 0.1mM 至约 10mM 的范围内。

29. 根据权利要求 26-28 中任一项所述的方法，其中所述至少一种生长调节剂包括胸苷。

30. 根据权利要求 29 所述的方法，其中所述胸苷的浓度在约 1mM 至约 100mM 的范围内。

31. 根据前述权利要求中任一项所述的方法，其中所述培养基具有在约 6.8-7.5 的范围内的 pH。

32. 根据权利要求 31 所述的方法，其中所述培养基具有在约 6.9-7.3 的范围内的 pH。

33. 根据前述权利要求中任一项所述的方法，其中所述培养步骤包括生长期和生产期。

34. 根据前述权利要求中任一项所述的方法，其中于在 30-37℃的范围内的温度下培养所述哺乳动物细胞。

35. 根据权利要求 33 或 34 所述的方法，其中在所述生长期和所述生产期期间在不同的温度下培养所述哺乳动物细胞。

36. 根据权利要求 33-35 中任一项所述的方法，其中用于所述生长期和所述生产期的所述培养基具有不同的 pH。

37. 根据权利要求 33-36 中任一项所述的方法，其中在所述生产期期间以在约 1.0-50X 10^6 个活细胞 /mL 的范围内的活细胞密度维持所述哺乳动物细胞。

38. 根据权利要求 33-37 中任一项所述的方法，其中所述生产期持续约 5-90 日。

39. 根据前述权利要求中任一项所述的方法，其中所述方法还包括收获所述重组 I2S 蛋白的步骤。

40. 根据前述权利要求中任一项所述的方法，其中所述重组 I2S 蛋白包含与 SEQ ID NO:1 具有至少 70％的同一性的氨基酸序列。

41. 根据权利要求 40 所述的方法，其中所述重组 I2S 蛋白包含与 SEQ ID NO:1 相同的氨基酸序列。

42. 根据前述权利要求中任一项所述的方法，其中所述 FGE 包含与 SEQ ID NO:5 具有至少 70％的同一性的氨基酸序列。

43. 根据权利要求 42 所述的方法，其中所述 FGE 包含与 SEQ ID NO:5 相同的氨基酸序列。

44. 根据前述权利要求中任一项所述的方法，其中所述细胞包含一个或多个编码所述重组 I2S 蛋白和 / 或所述 FGE 的外源核酸。

45. 根据权利要求 44 所述的方法，其中所述一个或多个外源核酸被整合到所述细胞的基因组中。

46. 根据权利要求 44 所述的方法，其中所述一个或多个外源核酸存在于一个或多个染色体外构建体上。

47. 根据前述权利要求中任一项所述的方法，其中所述细胞过表达所述重组 I2S 蛋白。

48. 根据前述权利要求中任一项所述的方法，其中所述细胞过表达所述 FGE。

49. 一种重组艾杜糖 2- 硫酸酯酶 (I2S) 蛋白，其利用根据前述权利要求中任一项所述的方法产生。

50. 一种重组艾杜糖 2- 硫酸酯酶 (I2S) 蛋白的制剂，所述重组 I2S 蛋白具有与 SEQ ID NO:1 具有至少 70％的同一性的氨基酸序列，并且包含至少约 70％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 C_{α}- 甲酰甘氨酸 (FGly) 的转化。

51. 根据权利要求 50 所述的制剂，其中所述重组 I2S 蛋白包含至少约 80％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 FGly 的转化。

52. 根据权利要求 50 或 51 所述的制剂，其中所述重组 I2S 蛋白包含至少约 90％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 FGly 的转化。

53. 根据权利要求 50-52 中任一项所述的制剂，其中所述重组 I2S 蛋白包含至少约 95％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 FGly 的转化。

54. 根据权利要求 50-53 中任一项所述的制剂，其中所述重组 I2S 蛋白包含至少约 97％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 FGly 的转化。

55. 根据权利要求 50-54 中任一项所述的制剂，其中所述重组 I2S 蛋白具有至少 40U/mg 的比活性，如通过使用肝素二糖作为底物的体外硫酸酯释放活性测定所测定的。

56. 根据权利要求 50-55 中任一项所述的制剂，其中所述重组 I2S 蛋白具有至少 60U/mg 的比活性，如通过使用肝素二糖作为底物的体外硫酸酯释放活性测定所测定的。

57. 根据权利要求 50-56 中任一项所述的制剂，其中所述重组 I2S 蛋白具有至少 80U/mg 的比活性，如通过使用肝素二糖作为底物的体外硫酸酯释放活性测定法所测定的。

58. 根据权利要求 50-57 中任一项所述的制剂，其中所述重组 I2S 蛋白包含与 SEQ ID NO:1 具有至少 70％的同一性的氨基酸序列。

59. 根据权利要求 50-58 中任一项所述的制剂，其中所述重组 I2S 蛋白包含与 SEQ ID NO:1 相同的氨基酸序列。

60. 一种药物组合物，其包含根据权利要求 50-59 中任一项所述的重组 I2S 蛋白和药学上可接受的载体。

61. 一种治疗亨特综合症的方法，所述方法包括向需要治疗的受试者施用根据权利要求 60 所述的药物组合物。

产生重组艾杜糖 -2- 硫酸酯酶的方法

[0001] 相关申请的交叉引用

[0002] 本申请要求 2012 年 6 月 29 日提交的美国临时专利申请系列号 61/666,712 的优先权 ;其完整内容在此通过引用并入。

[0003] 序列表

[0004] 在本说明书提及在 2013 年 6 月 27 日以电子形式提交的命名为“2006685-0339_SEQ_LIST”的 ASCII.txt 文件的序列表。所述 .txt 文件于 2013 年 6 月 25 日产生,并且大小为 21KB。将序列表的全部内容通过引用并入本文。

[0005] 背景

[0006] 粘多糖贮积症 II 型 (MPS II,亨特综合症) 是因酶艾杜糖 -2- 硫酸酯酶 (I2S) 的缺乏而引起的 X 染色体连锁隐性溶酶体贮积症。I2S 从葡糖氨基聚糖 (GAG) 硫酸皮肤素和硫酸乙酰肝素切割末端 2-O- 硫酸酯部分。由于亨特综合症患者中丧失 I2S 酶或存在有缺陷的 I2S 酶,因此 GAG 在多种细胞类型的溶酶体中逐渐累积,从而导致细胞肿胀,器官肿大,组织破坏和器官系统功能障碍。

[0007] 一般而言,具有亨特综合症的人的身体表现包括躯体和神经症状。例如,在亨特综合症的一些病例中,中枢神经系统受累导致发育迟缓和神经系统的问题。而亨特综合症的非神经症状在出生时一般不存在,随着时间的推移,GAG 在体内细胞中的渐进累积可能对身体的外周组织具有巨大影响。GAG 在外周组织中的累积导致患者面部特征的鲜明粗糙度,并且造成前额突出、扁平桥和巨舌,此为亨特综合征患者的最典型特征。类似地,GAG 的累积可不利地影响身体的器官系统。最初表现为心脏、肺和呼吸道的壁的增厚,以及肝、脾和肾的异常肿大,这些深刻的变化最终可导致广泛的灾难性器官衰竭。因此,亨特综合症总是严重的、进行性的和限制寿命的。

[0008] 酶替代疗法 (ERT) 是用于治疗亨特综合症 (MPS II) 的批准的疗法,其包括向亨特综合症患者施用外源替代 I2S 酶。

[0009] 发明概述

[0010] 本发明除其它事项以外提供了用于大规模产生重组 I2S 酶以促进亨特综合症的有效治疗的方法。在本发明之前,使用含血清培养基的滚瓶贴壁培养系统已被成功地开发来大规模产生重组 I2S。然而,本申请的发明人开发了这样的系统,该系统能使用无动物组分、化学成分确定的培养基在大规模器皿中有效地悬浮培养共表达 I2S 和甲酰甘氨酸生成酶 (FGE) 的哺乳动物细胞,以高效地产生大量重组 I2S 酶。出乎意料的是,使用无动物组分悬浮培养系统产生的重组 I2S 酶还已显著地改善了酶活性,因为以这种方式产生的重组 I2S 具有异常高水平的 C_{α}- 甲酰甘氨酸 (FGly)(例如,高于 70％和高达 100％),这是 I2S 的活性所需要的。此外,根据本发明产生的重组 I2S 酶具有独特的特征,例如唾液酸含量和聚糖图谱,这可以提高重组 I2S 蛋白的生物利用度。此外,无动物组分培养系统简化了下游纯化工艺,并减少或消除了血清来源的污染物如胎球蛋白。因此,本发明提供了大规模生产系统,该系统是更有效、具有成本效益、可重复的、更安全并产生更有效力的重组 I2S。

[0011] 因此,在一个方面,本发明提供了通过在含有缺乏血清的培养基的大规模培养皿

中悬浮培养共表达重组 I2S 蛋白和甲酰甘氨酸生成酶 (FGE) 的哺乳动物细胞而用于在哺乳动物细胞中大规模产生重组艾杜糖 -2- 硫酸酯酶 (I2S) 蛋白的方法。在一些实施方案中，培养步骤包括灌注工艺。

[0012] 在另一个方面，本发明提供了用于在哺乳动物细胞中大规模产生重组艾杜糖 -2- 硫酸酯酶 (I2S) 蛋白的方法，所述方法包括在一定条件下，在含有缺乏血清的培养基的大规模培养皿中培养共表达重组 I2S 蛋白和甲酰甘氨酸生成酶 (FGE) 的哺乳动物细胞，以便所述细胞平均以大于约 15 皮克 / 细胞 / 日的比生产率产生重组 I2S 蛋白，并且另外地其中产生的重组 I2S 蛋白平均包含至少约 60％的对应于人 I2S 蛋白的 Cys59 的半胱氨酸残基至 C_{α}- 甲酰甘氨酸的转化。在一些实施方案中，培养步骤包括灌注工艺。

[0013] 在一些实施方案中，灌注工艺具有在约 0.5-2 体积的新鲜培养基 / 反应器的工作体积 / 日 (VVD)(例如，约 0.5-1.5VVD、约 0.75-1.5VVD、约 0.75-1.25VVD、约 1.0-2.0VVD、约 1.0-1.9VVD、约 1.0-1.8VVD、约 1.0-1.7VVD、约 1.0-1.6VVD、约 1.0-1.5VVD、约 1.0-1.4VVD、约 1.0-1.3VVD、约 1.0-1.2VVD、约 1.0-1.1VVD) 的范围内的灌注速率。在一些实施方案中，灌注工艺具有约 0.5、0.55、0.6、0.65、0.7、0.75、0.8、0.85、0.9、0.95、1.0、1.05、1.10、1.15、1.2、1.25、1.3、1.35、1.4、1.45、1.5、1.55、1.6、1.65、1.7、1.75、1.8、1.85、1.9、1.95 或 2.0VVD 的灌注速率。

[0014] 在一些实施方案中，灌注工艺具有在约 0.05-5 纳升每种细胞每日 (nL/ 细胞 / 日)(例如，约 0.05-4nL/ 细胞 / 日、约 0.05-3nL/ 细胞 / 日、约 0.05-2nL/ 细胞 / 日、约 0.05-1nL/ 细胞 / 日、约 0.1-5nL/ 细胞 / 日、约 0.1-4nL/ 细胞 / 日、约 0.1-3nL/ 细胞 / 日、约 0.1-2nL/ 细胞 / 日、约 0.1-1nL/ 细胞 / 日、约 0.15-5nL/ 细胞 / 日、约 0.15-4nL/ 细胞 / 日、约 0.15-3nL/ 细胞 / 日、约 0.15-2nL/ 细胞 / 日、约 0.15-1nL/ 细胞 / 日、约 0.2-5nL/ 细胞 / 日、约 0.2-4nL/ 细胞 / 日、约 0.2-3nL/ 细胞 / 日、约 0.2-2nL/ 细胞 / 日、约 0.2-1nL/ 细胞 / 日、约 0.25-5nL/ 细胞 / 日、约 0.25-4nL/ 细胞 / 日、约 0.25-3nL/ 细胞 / 日、约 0.25-2nL/ 细胞 / 日、约 0.25-1nL/ 细胞 / 日、约 0.3-5nL/ 细胞 / 日、约 0.3-4nL/ 细胞 / 日、约 0.3-3nL/ 细胞 / 日、约 0.3-2nL/ 细胞 / 日、约 0.3-1nL/ 细胞 / 日、约 0.35-5nL/ 细胞 / 日、约 0.35-4nL/ 细胞 / 日、约 0.35-3nL/ 细胞 / 日、约 0.35-2nL/ 细胞 / 日、约 0.35-1nL/ 细胞 / 日、约 0.4-5nL/ 细胞 / 日、约 0.4-4nL/ 细胞 / 日、约 0.4-3nL/ 细胞 / 日、约 0.4-2nL/ 细胞 / 日、约 0.4-1nL/ 细胞 / 日、约 0.45-5nL/ 细胞 / 日、约 0.45-4nL/ 细胞 / 日、约 0.45-3nL/ 细胞 / 日、约 0.45-2nL/ 细胞 / 日、约 0.45-1nL/ 细胞 / 日、约 0.5-5nL/ 细胞 / 日、约 0.5-4nL/ 细胞 / 日、约 0.5-3nL/ 细胞 / 日、约 0.5-2nL/ 细胞 / 日、约 0.5-1nL/ 细胞 / 日) 的范围内的细胞比灌注速率。在一些实施方案中，灌注工艺具有约 0.05、0.1、0.15、0.2、0.25、0.3、0.35、0.4、0.45、0.5、0.55、0.6、0.65、0.7、0.75、0.8、0.85、0.9、0.95、1.0、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9、2.0、2.1、2.2、2.3、2.4、2.5、2.6、2.7、2.8、2.9、3.0、3.1、3.2、3.3、3.4、3.5、3.6、3.7、3.8、3.9、4.0、4.1、4.2、4.3、4.4、4.5、4.6、4.7、4.8、4.9 或 5.0nL/ 细胞 / 日的细胞比灌注速率。

[0015] 在一些实施方案中，根据本发明培养的细胞平均以大于约 20 皮克 / 细胞 / 日 (例如，大于约 25、30、35、40、45、50、55、60、65、70、75、80、85、90、95 或 100 皮克 / 细胞 / 日) 的比生产率产生重组 I2S 蛋白。在一些实施方案中，根据本发明培养的细胞以至少 6mg 每升每日 (mg/L/ 日)(例如，至少 8、10、12、14、16、18、20、25、30、35、40、45、50、55、60、65、70、75、

80、85、90、95、100、150、200、250、300、350、400、450 或 500mg/L/ 日或更多）的平均收获滴度产生重组 I2S 蛋白。

[0016] 在一些实施方案中，根据本发明的方法的产生的重组 I2S 蛋白包含至少约 70％（例如，至少约 75％、80％、85％、90％、95％、96％、97％、98％、99％、100％）的对应于人 I2S 蛋白的 Cys59 的半胱氨酸残基至 C_{α}- 甲酰甘氨酸 (FGly) 的转化。

[0017] 在一些实施方案中，适合于本发明的哺乳动物细胞为人细胞。在一些实施方案中，适合于本发明的哺乳动物细胞为 CHO 细胞。

[0018] 在一些实施方案中，适合于本发明的大规模培养皿为生物反应器。在一些实施方案中，适合的生物反应器为或大于 10L、200L、500L、1000L、1500L、2000L、2500L、3000L 的规模。

[0019] 在一些实施方案中，适合于本发明的培养基缺乏动物来源的组分。在一些实施方案中，适合的培养基是化学成分确定的培养基。在一些实施方案中，适合的培养基不含蛋白质。

[0020] 在一些实施方案中，适合于本发明的培养基包含至少一种氧化还原调节剂。在一些实施方案中，适合于本发明的氧化还原调节剂选自谷胱甘肽、葡萄糖 -6- 磷酸、肌肽、鼠尾草酚、萝卜硫素、生育酚、抗坏血酸、脱氢抗坏血酸、硒、2- 巯基乙醇、N- 乙酰半胱氨酸、半胱氨酸、核黄素、烟酸、叶酸、黄素腺嘌呤二核苷酸 (FAD)、烟酰胺腺嘌呤二核苷酸磷酸 (NADP) 及其组合。在一些实施方案中，适合的氧化还原调节剂为半胱氨酸。在一些实施方案中，半胱氨酸的浓度在约 0.1mg/L 至约 65mg/L（例如，1-50mg/L、1-40mg/L、1-30mg/l、1-20mg/L、1-10mg/L）的范围内。在一些实施方案中，适合的氧化还原调节剂为 2- 巯基乙醇。在一些实施方案中，2- 巯基乙醇的浓度在约 0.001mM 至约 0.01mM（例如，约 0.001-0.008mM、约 0.001-0.007mM、约 0.001-0.006mM、约 0.001-0.005mM、约 0.001-0.004mM、约 0.001-0.003mM、约 0.001-0.002mM）的范围内。在一些实施方案中，适合的氧化还原调节剂为 N- 乙酰半胱氨酸。在一些实施方案中，N- 乙酰半胱氨酸的浓度在约 3mM 至约 9mM（例如，约 3-8mM、约 3-7mM、约 3-6mM、约 3-5mM、约 3-4mM）的范围内。

[0021] 在一些实施方案中，适合本发明的培养基包含至少一种生长调节剂。在一些实施方案中，适合的生长调节剂为次黄嘌呤。在一些实施方案中，次黄嘌呤的浓度在约 0.1mM 至约 10mM（例如，约 0.1-9mM、约 0.1-8mM、约 0.1-7mM、约 0.1-6mM、约 0.1-5mM、约 0.1-4mM、约 0.1-3mM、约 0.1-2mM、约 0.1-1mM）的范围内。在一些实施方案中，适合的生长调节剂为胸苷。在一些实施方案中，胸苷的浓度在约 1mM 至约 100mM（例如，约 1-90mM、约 1-80mM、约 1-70mM、约 1-60mM、约 1-50mM、约 1-40mM、约 1-30mM、约 1-20mM、约 1-10mM）的范围内。

[0022] 在一些实施方案中，培养基具有在约 6.8 - 7.5（例如，约 6.9-7.4、约 6.9-7.3、约 6.95-7.3、约 6.95-7.25、约 7.0-7.3、约 7.0-7.25、约 7.0-7.2、约 7.0-7.15、约 7.05-7.3、约 7.05-7.25、约 7.05-7.15、约 7.05-7.20、约 7.10-7.3、约 7.10-7.25、约 7.10-7.20、约 7.10-7.15）的范围内的 pH。在一些实施方案中，培养基具有约 6.8、6.85、6.9、6.95、7.0、7.05、7.1、7.15、7.2、7.25、7.3、7.35、7.4、7.45 或 7.5 的 pH。

[0023] 在一些实施方案中，本文中描述的各种方法的培养步骤包括生长期和生产期。在一些实施方案中，于在约 30-37℃（例如，约 31-37℃、约 32-37℃、约 33-37℃、约 34-37℃、约 35-37℃、约 36-37℃）的范围内的温度下培养哺乳动物细胞。在一些实施方案中，在约

30℃、31℃、32℃、33℃、34℃、35℃、36℃或37℃的温度下培养哺乳动物细胞。本文中描述的任何温度可用于生长和/或生产期。在一些实施方案中，在生长期和生产期期间在不同的温度下培养哺乳动物细胞。在一些实施方案中，在生长期和生产期期间在大体上相同的温度下培养哺乳动物细胞。本文中描述的任何培养基pH可用于生长和/或生产期。在一些实施方案中，对于生长期和生产期的培养基pH是不同的。在一些实施方案中，对于生长期和生产期的培养基pH是大体上相同的。

[0024] 在一些实施方案中，在生产期期间以在约1.0-50X10^{6}个活细胞/mL(例如，约1.0-40X10^{6}个活细胞/mL、约1.0-30X10^{6}个活细胞/mL、约1.0-20X10^{6}个活细胞/mL、约1.0-10X10^{6}个活细胞/mL、约1.0-5X10^{6}个活细胞/mL、约1.0-4.5X10^{6}个活细胞/mL、约1.0-4X10^{6}个活细胞/mL、约1.0-3.5X10^{6}个活细胞/mL、约1.0-3X10^{6}个活细胞/mL、约1.0-2.5X10^{6}个活细胞/mL、约1.0-2.0X10^{6}个活细胞/mL、约1.0-1.5X10^{6}个活细胞/mL、约1.5-10X10^{6}个活细胞/mL、约1.5-5X10^{6}个活细胞/mL、约1.5-4.5X10^{6}个活细胞/mL、约1.5-4X10^{6}个活细胞/mL、约1.5-3.5X10^{6}个活细胞/mL、约1.5-3.0X10^{6}个活细胞/mL、约1.5-2.5X10^{6}个活细胞/mL、约1.5-2.0X10^{6}活细胞/mL)的范围内的活细胞密度维持哺乳动物细胞。

[0025] 在一些实施方案中，生产期持续约5-90日(例如，约5-80日、约5-70日、约5-60日、约5-50日、约5-40、约5-30日、约5-20日、约5-15日、约5-10日、约10-90日、约10-80日、约10-70日、约10-60日、约10-50日、约10-40日、约10-30日、约10-20日、约15-90日、约15-80日、约15-70日、约15-60日、约15-50日、约15-40日、约15-30日)。在一些实施方案中，生产期持续约5、10、15、20、25、30、35、40、45、50、55、60、65、70、75、80、85或90日。

[0026] 在各个实施方案中，哺乳动物细胞表达具有与SEQ ID NO:1具有至少约50%(例如，至少约55%、60%、65%、70%、75%、80%、85%、90%、95%、96%、97%、98%、99%)的同一性的氨基酸序列的重组I2S蛋白。在一些实施方案中，本文中描述的发明方法用于产生具有与SEQ ID NO:1相同的氨基酸序列的重组I2S蛋白。

[0027] 在各个实施方案中，哺乳动物细胞表达具有与SEQ ID NO:5具有至少约50%(例如，至少约55%、60%、65%、70%、75%、80%、85%、90%、95%、96%、97%、98%、99%)的同一性的氨基酸序列的FGE蛋白。在一些实施方案中，哺乳动物细胞表达具有与SEQ ID NO:5相同的氨基酸序列的FGE蛋白。

[0028] 在各个实施方案中，哺乳动物细胞包含一个或多个编码重组I2S蛋白和/或FGE的外源核酸。在一些实施方案中，将一个或多个外源核酸整合到细胞的基因组中。在一些实施方案中，一个或多个外源核酸存在于一个或多个染色体外构建体上。在一些实施方案中，用于本发明的方法的哺乳动物细胞过表达重组I2S蛋白。在一些实施方案中，用于本发明的方法的哺乳动物细胞过表达FGE。

[0029] 在各个实施方案中，根据本发明的发明方法还包括收获重组I2S蛋白的步骤。

[0030] 在另一个方面，本发明提供了使用本文中描述的方法产生的重组艾杜糖2-硫酸酯酶(I2S)蛋白。在一些实施方案中，本发明提供了重组I2S蛋白的制剂，其中重组I2S蛋白具有至少约70%(例如，至少约77%、80%、85%、90%、95%、96%、97%、98%、99%)的对应于人I2S(SEQ ID NO:1)的Cys59的半胱氨酸残基至C_α-甲酰甘氨酸(FGly)的转化。

在一些实施方案中，本发明提供了重组 I2S 蛋白的制剂，其中重组 I2S 蛋白具有大体 100％的对应于人 I2S(SEQ ID NO:1) 的 Cys59 的半胱氨酸残基至 C_{α}- 甲酰甘氨酸 (FGly) 的转化。在一些实施方案中，本发明提供了重组艾杜糖 2- 硫酸酯酶 (I2S) 蛋白的制剂，所述重组 I2S 蛋白具有与 SEQ ID NO:1 具有至少约 50％（例如，至少约 55％、60％、65％、70％、75％、80％、85％、90％、95％、96％、97％、98％、99％）的同一性的氨基酸序列。在一些实施方案中，重组 I2S 蛋白具有与 SEQ ID NO:1 相同的氨基酸序列。

[0031] 在一些实施方案中，重组 I2S 蛋白具有至少约 20U/mg、30U/mg、40U/mg、50U/mg、60U/mg、70U/mg、80U/mg、90U/mg 或 100U/mg 的比活性，如通过使用肝素二糖作为底物的体外硫酸酯释放活性测定所测定的。

[0032] 除其它事项以外，本发明还提供了一种包含本文中各个实施方案中描述的重组 I2S 蛋白和药学上可接受的载体的药物组合物，以及通过向需要治疗的受试者施用本文中描述的重组 I2S 蛋白或包含所述重组 I2S 蛋白的药物组合物来治疗亨特综合症的方法。

[0033] 如本文中所用，除非另有明确所指，否则术语“I2S 蛋白”、“I2S”、“I2S 酶”或语法等同物是指重组 I2S 蛋白分子的制剂。

[0034] 如本申请中所用，术语“约”和“大致”可作为等同物使用。具有或不具有约 / 大致的用于本申请的任何数字旨在涵盖由相关领域的普通技术人员所理解的任何正常波动。

[0035] 本发明的其他特征、目的和优势在下面的详细描述中显而易见。然而，应当理解，所述详细描述，虽然表示本发明的实施方案，但仅通过举例说明的方式给出，而非限制性的。根据所述详细描述，本发明的范围内的各种变化和修改对于本领域技术人员来说是显而易见的。

[0036] 附图简述

[0037] 共同地构成附图的以下描述的图仅用于说明目的而不是为了限制。

[0038] 图 1 描述了编码人艾杜糖 -2- 硫酸酯酶 (I2S) 蛋白的成熟形式的氨基酸序列 (SEQ ID NO:1)，并且指示了蛋白质序列内用于 N 连接糖基化和半胱氨酸转化的潜在位点。

[0039] 图 2 描述了用于共表达 I2S 和 FGE 的示例性构建体（即，SUMF1）设计。(A) 分开的载体上的表达单位（用于共转染或随后的转染）；(B) 相同载体上的表达单位（一次转染）：(1) 分隔的顺反子和 (2) 转录连接的顺反子。

[0040] 图 3 显示了相较于 I2S 参考标准，通过使用在无血清或基于血清的细胞培养条件下生长的细胞系产生的 SDS-PAGE 显示的全长重组 I2S 的示例性表达。

[0041] 图 4 显示从在无血清培养条件（顶图）下生长的 I2S-AF 2D 细胞系产生的重组 I2S 酶相对参考重组 I2S 酶的示例性肽图谱

[0042] 图 5 描述了相较于参考重组 I2S 酶，针对使用在无血清细胞培养条件下生长的 I2S-AF 2D 和 4D 细胞系产生的重组 I2S 酶产生的示例性聚糖特征谱。

[0043] 图 6 描述了相较于参考重组 I2S 酶，针对使用在无血清细胞培养条件下生长的 I2S-AF 2D 细胞系产生的重组 I2S 酶产生的示例性电荷特征谱。

[0044] 定义

[0045] 为了使本发明更容易被理解，首先定义某些术语。对下列术语和其他术语的附加定义陈述于整个说明书中。

[0046] 氨基酸：如本文中所用，术语“氨基酸”在其最广泛意义上是指可并入多肽链中的

任何化合物和/或物质。在一些实施方案中，氨基酸具有一般结构 $H_2N-C(H)(R)-COOH$。在一些实施方案中，氨基酸是天然存在的氨基酸。在一些实施方案中，氨基酸是合成氨基酸；在一些实施方案中，氨基酸是 D- 氨基酸；在一些实施方案中，氨基酸是 L- 氨基酸。“标准氨基酸”是指通常在天然存在的肽中发现的 20 种标准 L- 氨基酸中的任一种。“非标准氨基酸”是指除标准氨基酸外的任何氨基酸，无论其是合成制备的还是从天然来源获得的。如本文中所用，“合成氨基酸”涵盖化学修饰氨基酸，包括但不限于盐，氨基酸衍生物（如酰胺）和/或取代。氨基酸，包括在肽中的羧基和/或氨基末端氨基酸，可通过甲基化、酰胺化、乙酰化、保护基团和/或利用其他化学基团的取代来进行修饰，所述其他化学基团可改变肽的循环半衰期而不会不利地影响它们的活性。氨基酸可参与二硫键。氨基酸可以包含一种或多种翻译后修饰，例如与一种或多种化学实体（例如，甲基、乙酸酯基、乙酰基、磷酸酯基、甲酰基部分、类异戊二烯基、硫酸酯基、聚乙二醇部分、脂质部分、碳水化合物部分、生物素部分等的缔合。在一些实施方案中，可在用于细胞培养的补充培养基中提供本发明的氨基酸，或可将所述氨基酸用于所述补充培养基。在一些实施方案中，提供于或用于补充细胞培养基中的氨基酸可以以盐或水合物的形式提供。

[0047] 大致：如本文中所用，术语“大致”或“约”，如用于一个或多个目标值，意指与所述参照值相似的值。在某些实施方案中，除非另外指出或另外地根据上下文显而易见的（除其中这样的数值超过可能值的 100％外），否则术语“大致”或“约”是指在任一方向（大于或小于）上落在所述参照值的 25％、20％、19％、18％、17％、16％、15％、14％、13％、12％、11％、10％、9％、8％、7％、6％、5％、4％、3％、2％、1％或更少内的值的范围。

[0048] 分批培养：如本文中所用，术语“分批培养”是指培养细胞的方法，在所述方法中在培养过程开始时提供最终将用于培养细胞的所有组分，包括培养基（参见下文中“培养基”的定义）以及细胞本身。因此，分批培养通常是指允许从接种进展至结束而无需给培养的细胞重新进料新鲜培养基的培养。通常在某一点停止分批培养，随后收获培养基中细胞和/或组分，并且任选地进行纯化。

[0049] 生物利用度：如本文中所用，术语“生物利用度”通常是指到达受试者的血流的施用剂量的百分比。

[0050] 生物活性：如本文中所用，短语“生物活性”是指在生物系统（例如，细胞培养物、生物体等）中具有活性的任何物质的特征。例如，当向生物体施用时对该生物体具有生物作用的物质被认为是有生物活性的。生物活性还可通过体外测定（例如，体外酶促测定如硫酸酯释放测定法）来确定。在具体实施方案中，当蛋白质或多肽具有生物学活性时，共有蛋白质或多肽的至少一种生物活性的该蛋白质或多肽的一部分通常称为“生物活性”部分。在一些实施方案中，从细胞培养系统产生和/或纯化蛋白质，当向受试者施用时，所述蛋白质显示生物活性。在一些实施方案中，蛋白质需要进一步加工以变得具有生物活性。在一些实施方案中，蛋白质需要翻译后修饰，例如但不限于，糖基化（例如，唾液酸化(sialyation)）、法尼基化(farnysylation)、切割、折叠、甲酰甘氨酸转化及其组合，以变得具有生物活性。在一些实施方案中，作为前体(proform)形式（即未成熟的形式）产生的蛋白质可能需要额外的修饰来变得具有生物活性。

[0051] 生物反应器：如本文所用，术语“生物反应器”是指用于宿主细胞培养物生长的容器。生物反应器可具有任何尺寸，只要它对于哺乳动物细胞的培养是有用的。通常情况下，

生物反应器将至少为 1 升，并且可以是 10、100、250、500、1000、2500、5000、8000、10,000、12,0000 升或以上或其间的任何容积。生物反应器的内部条件，包括但不限于 pH 值、克分子渗透压浓度、CO_2饱和度、O_2饱和度、温度及其组合，在培养期间通常受到控制。生物反应器可由适合于在本发明的培养条件下在培养基中保持细胞的任何材料（包括玻璃、塑料或金属）组成。在一些实施方案中，生物反应器可用于进行动物细胞培养。在一些实施方案中，生物反应器可用于进行哺乳动物细胞培养。在一些实施方案中，可将生物反应器与来源于这样的生物体的细胞和 / 或细胞系（例如但不限于哺乳动物细胞、昆虫细胞、细菌细胞、酵母细胞和人细胞）一起使用。在一些实施方案中，生物反应器可用于大规模细胞培养生产，并且通常为至少 100 升，并且可以为 200、500、1000、2500、5000、8000、10,000、12,0000 升或以上或其间的任何容。本领域普通技术人员将意识到，并且将能够选择适合的生物反应器用于实施本发明。

[0052] 细胞密度：如本文中所用，术语“细胞密度”是指存在于给定体积的培养基中的细胞的数目。

[0053] 细胞培养物或培养物：如本文中所用，这些术语是指在适合于细胞群体存活和 / 或生长的条件下在培养基中生长的细胞群。如对于本领域普通技术人员将很清楚的，如本文中使用的这些术语可指包含细胞群和所述群于其中生长的培养基的组合。

[0054] 培养：如本文中所用，术语“培养”或语法等同物是指在有利于生长或存活的条件下维持细胞的过程。术语“培养”和“细胞培养”或任何同义词在本申请中可互换使用。

[0055] 培养皿：如本文中所用，术语“培养皿”是指可为培养细胞提供无菌环境的任何容器。示例性培养皿包括但不限于玻璃、塑料或金属容器。

[0056] 剂型：如本文中所用，术语“剂型”和“单位剂型”是指用于待治疗的患者的治疗性蛋白的物理上分离的单位。每一个单位包含经计算产生期望的疗效的预定量的活性材料。然而，应理解，组合物的总剂量将由健全医学范围内的主治医生来确定。

[0057] 给药方案：如该术语在本文中所用的，“给药方案”（或“治疗方案”）是向受试者单独施用（通常间隔以时间段）的一组单位剂量（通常超过 1 个）。在一些实施方案中，给定的治疗剂具有推荐的给药方案，其可包括一个或多个剂量。在一些实施方案中，给药方案包括多个剂量，其每一个剂量彼此间隔相同长度的一段时间；在一些实施方案中，给药方案包括多个剂量和至少两个不同的间隔单个剂量的时间段。

[0058] 酶替代疗法 (ERT)：如本文中所用，术语“酶替代疗法 (ERT)”指的是通过提供缺失的酶校正酶缺乏的任何治疗策略。在一些实施方案中，缺失的酶通过鞘内施用提供。在一些实施方案中，缺失的酶通过向血流内的输注提供。一旦施用，酶被细胞吸收并转运至溶酶体，在溶酶体中酶用于消除因酶缺乏而在溶酶体中积累的物质。通常情况下，为了使溶酶体酶替代疗法有效，将治疗性酶递送至其中表现贮积缺陷的靶组织中适合的细胞中的溶酶体中。

[0059] 赋形剂：如本文中所用，术语“赋形剂”是指被添加至药物和 / 或制剂以改善其物理性质（即稠度）、药代动力学性质（即生物利用度）、药效动力学性质及其组合的任何惰性物质。

[0060] 表达：如本文中所用，核酸序列的“表达”是指下列事件的一个或多个：(1)RNA 模板从 DNA 序列的产生（例如，通过转录）；(2)RNA 转录物的加工（例如，通过剪接、编辑、5’

帽形成和 / 或 3’ 末端形成 ）；(3)RNA 至多肽或蛋白质的翻译；和 / 或 (4) 多肽或蛋白质的翻译后修饰。

[0061] 补料分批培养：如本文所用，术语“补料分批培养”是指培养细胞的方法，在所述方法中，在培养过程开始后的某个时间给培养物提供额外的组分。所提供的组分通常包括已在培养过程中被耗尽的细胞的营养补充剂。通常在某个点停止补料分批培养，并且收获培养基中的细胞和 / 或组分和任选地纯化其。

[0062] 片段：如本文中所用，术语“片段”是指多肽，并且被定义为给定的多肽的任何分离的部分，所述部分对于该多肽是独特的，或具有该多肽的特征。如本文中所用，该术语还指给定的多肽的保留了全长多肽的至少一部分活性的任何分离的部分。优选地，保留的活性的部分为全长多肽的活性的至少 10％。更优选，保留的活性的部分为全长多肽的活性的至少 20％、30％、40％、50％、60％、70％、80％或 90％。更优选，保留的活性的部分为全长多肽的活性的至少 95％、96％、97％、98％或 99％。最优选，保留的活性的部分为全长多肽的活性的 100％。如本文中所用，该术语还指包含至少在全长多肽中发现的已建立的序列元件的给定的多肽的任何部分。优选地，序列元件跨越全长多肽的至少 4-5，更优选至少约 10、15、20、25、30、35、40、45、50 或更多个氨基酸。

[0063] 基因：如本文中所用，术语“基因”是指任何核苷酸序列 DNA 或 RNA，所述核苷酸序列的至少某一部分编码分开的终产物，通常地（但不限于）多肽，所述产物在细胞过程的某一方面起作用。该术语并不意味仅指编码多肽或其它分离的终产物的编码序列，而且还可包括调节基础表达水平的编码序列之前和之后的区域，以及各个编码区段（“外显子”）之间的间插序列（“内含子”）。在一些实施方案中，基因可包括调节序列（例如，启动子、增强子、聚腺苷酸化序列、终止序列、Kozak 序列、TATA 盒等）和 / 或修饰序列。在一些实施方案中，基因可包括对不编码蛋白质但相反地编码功能性 RNA 分子例如 tRNA 基因、RNAi 诱导剂等的核酸的提及。

[0064] 基因产物或表达产物：如本文中所用，术语“基因产物”或“表达产物”一般是指从基因转录的 RNA（加工前和 / 或加工后）或由从基因转录的 RNA 编码的多肽（修饰前和 / 或修饰后）。

[0065] 基因控制元件：如本文中所用，术语“基因控制元件”是指调节与其可操作地连接的基因的表达的任何序列元件。基因控制元件可通过升高或降低表达水平来起作用，并且可位于编码序列之前、之内或之后。基因控制元件可通过调节例如转录的起始、延伸或终止、mRNA 剪接、mRNA 编辑、mRNA 稳定性、细胞内 mRNA 的定位、翻译的起始、延伸或终止而在基因表达的任何阶段起作用或在基因表达的任何其它阶段起作用。基因控制元件可单独地或彼此组合地起作用。

[0066] 同源性：如本文中所用，术语“同源性”是指聚合分子之间，例如核酸分子（例如，DNA 分子和 / 或 RNA 分子）之间和 / 或多肽分子之间的总体相关性。在一些实施方案中，如果聚合分子的序列具有至少 25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％或 99％的同一性，则它们被认为是彼此“同源的”。在一些实施方案中，如果聚合分子的序列具有至少 25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％或 99％的相似性，则它们被认为是彼此“同源的”。

[0067] 同一性：如本文中所用，“同一性”是指聚合分子之间，例如核酸分子（例如，DNA

分子和/或RNA分子）之间和/或多肽分子之间的总体相关性。两个核酸序列的百分比同一性的计算，例如，可通过为了最佳比较目的而比对两个序列（例如，可在第一和第二核酸序列的一个或两个中引入缺口来进行最佳比对，并且为了比较目的可忽略不相同的序列）来进行。在某些实施方案中，为了比较目的而比对的序列的长度为参照序列的长度的至少30％，至少40％，至少50％，至少60％，至少70％，至少80％，至少90％，至少95％或大体上100％。随后比较对应核苷酸位置上的核苷酸。当第一序列中的位置被与第二序列中的对应位置相同的核苷酸占据时，则分子在该位置上是相同的。通过考虑两个序列的最佳比对而需要引入的缺口的数目和每个缺口的长度，两个序列之间的同一性百分比是序列共有的相同位置的数目的函数。序列的比较和两个序列之间的同一性百分比的测定可使用数学算法来实现。例如，例如，两个核苷酸序列之间的同一性百分比可使用Meyers和Miller的算法(CABIOS,1989,4:11-17)，使用PAM120权重残基表、缺口长度罚分12和缺口罚分4来测定，所述算法已被整合至ALIGN程序(2.0版）中。或者，可使用GCG软件包中的GAP程序，使用NWSgapdna.CMP矩阵来测定两个核苷酸序列之间的同一性百分比。各种其它序列比对程序是可用的，并且可被用来测定序列同一性，例如，Clustal。

[0068] 提高、增加或减少：如本文中所用，术语“提高”、“增加”或“减少”或语法等同物，指示相对于基线测量，例如在本文中描述的治疗开始之前相同个体中的测量或在本文中描述的治疗不存在的情况下对照个体（或多个对照个体）中的测量的值。“对照个体”是患有与正在治疗的个体相同的溶酶体贮积病形式的个体，所述个体与正在治疗的个体年龄大致相同（以确保被治疗个体与对照个体的疾病分期是可比较的）。

[0069] 积分活细胞密度：如本文中所用，术语“积分活细胞密度”是指整个培养过程中活细胞的平均密度乘以培养已进行的时间的量。假定产生的多肽或蛋白质的量与整个培养过程中存在的活细胞的数目成正比，则积分活细胞密度是用于估计整个培养过程中产生的多肽和/或蛋白质的量的有用工具。

[0070] 鞘内施用：如本文中所用，术语“鞘内施用”或“鞘内注射”是指至椎管内的注射（围绕脊髓的鞘内空间）。可使用各种技术，包括但不限于，通过钻孔的侧脑室注射或池穿刺或腰椎穿刺等。在一些实施方案中，根据本发明的“鞘内施用”或“鞘内递送”是指通过腰部区域或腰部区的IT施用或递送，即腰IT施用或递送。如本文中所用，术语“腰部区”或“腰部区域”指的是第三和第四腰（下背部）椎骨之间的区域，更包含地，脊椎的L2-S1区。

[0071] 分离的：如本文中所用，术语“分离的”是指这样的物质和/或实体，所述物质和/或实体(1)已与当最初产生（无论是在自然界中还是在实验环境中）时与其结合的组分的至少一些分离，和/或(2)已通过人的手产生、制备和/或制造。分离的物质和/或实体可与约10％、约20％、约30％、约40％、约50％、约60％、约70％、约80％、约90％、约91％、约92％、约93％、约94％、约95％、约96％、约97％、约98％、约99％或更多的与它们最初结合的其它组分分离。在一些实施方案中，分离物质具有约80％、约85％、约90％、约91％、约92％、约93％、约94％、约95％、约96％、约97％、约98％、约99％或高于约99％的纯度。如本文中所用，如果物质基本上不含其它组分的话，则它是“纯的”。如本文中所用，分离的物质和/或实体的百分比纯度的计算不应包括赋形剂（例如，缓冲液、溶剂、水等）。

[0072] 培养基：如本文中所用，该术语指包含有素滋养生长细胞的营养物的溶液。通常情况下，这些溶液提供了细胞进行最低生长和/或存活所需的必需和非必需氨基酸、维生素、

能量来源、脂质和微量元素。该溶液还可包含增强高于最小速率的生长和/或存活的组分，包括激素和生长因子。在一些实施方案中，将培养基配制为对于细胞存活和增殖是最佳的pH和盐浓度。在一些实施方案中，培养基可以是“化学成分确定的培养基”—不含蛋白质、水解产物或未知组合物的组分的无血清培养基。在一些实施方案中，化学成分确定的培养基不含动物来源的组分并且培养基中的所有组分具有已知的化学结构。在一些实施方案中，培养基可以是“基于血清的培养基”—已补充了动物来源的组分例如但不限于胎牛血清、马血清、山羊血清、驴血清和/或其组合的培养基。

[0073] 代谢废产物：如本文所用，术语“代谢废产物”是指由细胞培养物作为正常或不正常的代谢过程的结果产生的化合物，所述化合物在某种程度上对细胞培养物是有害的，尤其是关乎期望的重组多肽或蛋白质的表达或活性。例如，代谢废产物可对细胞培养物的生长或活力有害，可减少产生的重组多肽或蛋白质的量，可改变表达的多肽或蛋白质的折叠、稳定性、糖基化或其它翻译后修饰，或可以以许多其它方式损害细胞和/或重组多肽或蛋白质的表达。示例性代谢废产物包括乳酸（其由葡萄糖代谢产生）和铵（其由谷氨酰胺代谢产生）。本发明的一个目的是在哺乳动物细胞培养物中减缓代谢废产物的产生，减少或甚至消除代谢废产物。

[0074] 核酸：如本文中所用，术语“核酸”以其最广泛的意义是指为寡核苷酸链或可被并入寡核苷酸链的化合物和/或物质。在一些实施方案中，核酸是作为寡核苷酸链或可通过磷酸二酯键并入寡核苷酸链的化合物和/或物质。在一些实施方案中，“核酸”是指个别核酸残基（例如，核苷酸和/或核苷）。在一些实施方案中，“核酸”是指包含个别核酸残基的寡核苷酸链。如本文所使用的，术语“寡核苷酸”和“多核苷酸”可以互换使用。在一些实施方案中，“核酸”涵盖RNA以及单链和/或双链DNA和/或cDNA。此外，术语“核酸”、“DNA”、“RNA”和/或类似术语包括核酸类似物，即，具有除磷酸二酯骨架外的类似物。例如，所谓的“肽核酸”，其在本领域是已知的并且在骨架中具有肽键而非磷酸二酯键，被认为在本发明的范围之内。术语“编码氨基酸序列的核苷酸序列”包括互为简并形式和/或编码相同氨基酸序列的所有核苷酸序列。编码蛋白质和/或RNA的核苷酸序列可包括内含子。核酸可以从天然来源中纯化，使用重组表达系统产生并任选地纯化，化学合成等。在适当情况下，例如，在化学合成分子的情况下，核酸可包含核苷类似物，例如具有经化学修饰的碱基或糖、骨架修饰等的类似物。除非另有说明，否则核酸序列以5’至3’方向显示。术语“核酸区段”在本文中用于指作为更长的核酸序列的一部分的核酸序列。在许多实施例中，核酸区段包含至少3、4、5、6、7、8、9、10或更多个残基。在一些实施方案中，核酸是或包含天然核苷（例如，腺苷、胸苷、鸟苷、胞苷、尿苷、脱氧腺苷、脱氧胸苷、脱氧鸟苷和脱氧胞苷）；核苷类似物（例如，2-氨基腺苷、2-硫代胸苷、肌苷、吡咯并嘧啶、3-甲基腺苷、5-甲基胞苷、C-5丙炔基-胞苷、C-5丙炔基-尿苷、2-氨基腺苷、C5-溴尿苷、C5-氟尿苷、C5-碘尿苷、C5-丙炔基-尿苷、C5-丙炔基-胞苷、C5-甲基胞苷、2-氨基腺苷、7-脱氮腺苷、7-脱氮鸟苷、8-氧代腺苷、8-氧代鸟苷、O(6)-甲基鸟嘌呤和2-硫代胞苷）；化学修饰碱基；生物修饰的碱基（例如，甲基化碱基）；嵌入碱基；修饰糖（例如，2’-氟代核糖、核糖、2’-脱氧核糖、阿拉伯糖和己糖）；和/或修饰磷酸基团（例如，硫代磷酸酯和5’-N-亚磷酰胺键）。在一些实施方案中，本发明特别针对“未修饰核酸”，意指未经历化学修饰以促进或实现递送的核酸（例如，多核苷酸和残基，包括核苷酸和/或核苷）。

[0075] 克分子渗透压浓度和摩尔渗透压浓度：“摩尔渗透压浓度”是水溶液中溶解的溶质粒子的渗透压的量度。溶质粒子包括离子和非电离分子。摩尔渗透压浓度表达为溶解在1kg溶液中的渗透活性粒子的浓度（即，渗透压摩尔）(1mOsm/kg H_2O 在38℃相当于19mm Hg的渗透压）。相比之下，″克分子渗透压浓度″是指溶解在1升溶液中的溶质粒子的数目。当在本文中使用时，缩写″mOsm″意指″毫渗透摩尔/kg溶液″。

[0076] 灌注工艺：如本文中所用，术语“灌注工艺”是指其中在培养过程开始之后将另外的组分连续地或半连续地提供给培养物的培养细胞的方法。所提供的组分通常包括已在培养过程中被耗尽的细胞的营养补充剂。通常在连续或半连续的基础上收获培养基中的一部分细胞和/或组分并且任选地对其进行纯化。通常情况下，涉及灌注工艺的细胞培养过程被称为“灌注培养”。通常情况下，在灌注工艺过程中在新鲜培养基中提供营养补充剂。在一些实施方案中，新鲜培养基可与细胞培养过程中使用的基本培养基相同或相似。在一些实施方案中，新鲜的培养基可与基本培养基不同，但包含期望的营养补充剂。在一些实施方案中，新鲜培养基是化学成分确定的培养基。

[0077] 蛋白质：如本文中所用，术语“蛋白质”是指多肽（即，至少2个通过肽键彼此连接的氨基酸的串）。蛋白质可包括除氨基酸外的部分（例如，可以是糖蛋白、蛋白聚糖等）和/或可另外地被加工或修饰。本领域普通技术人员将理解，“蛋白质”可以是如由细胞产生的完整的多肽链（具有或不具有信号序列），或可为其特征部分。在一些实施方案中，蛋白质有时可包括1个以上的多肽链，例如，通过一个或多个二硫键连接或通过其它方式缔合。在一些实施方案中，多肽可含有L-氨基酸、D-氨基酸或两者，并且可以包含多种本领域已知的氨基酸修饰或类似物中的任一种。有用的修饰包括，例如，末端乙酰化、酰胺化、甲基化等。在一些实施方案中，蛋白质可包含天然氨基酸、非天然氨基酸、合成氨基酸及其组合。术语“肽”通常用于指具有长度少于约100个氨基酸、少于约50个氨基酸、少于20个氨基酸或少于10个氨基酸的肽。在一些实施方案中，蛋白质是抗体、抗体片段、其生物活性部分和/或其特征部分。

[0078] 重组蛋白和重组多肽：如本文中所用，这些术语是指从宿主细胞表达的多肽，所述宿主细胞已经被基因工程化以表达该多肽。在一些实施方案中，重组蛋白可在来源于动物的宿主细胞中表达。在一些实施方案中，重组蛋白可在来源于昆虫的宿主细胞中表达。在一些实施方案中，重组蛋白可在来源于酵母的宿主细胞中表达。在一些实施方案中，重组蛋白可在来源于原核生物的宿主细胞中表达。在一些实施方案中，重组蛋白可在来源于哺乳动物的宿主细胞中表达。在一些实施方案中，重组蛋白可在来源于人的宿主细胞中表达。在一些实施方案中，所述重组表达的多肽可与在宿主细胞中正常表达的多肽相同或相似。在一些实施方案中，重组表达的多肽对于宿主细胞可以是外来的，即对于在宿主细胞中正常表达的肽是异源的。或者，在一些实施方案中，重组表达的多肽可以是嵌合的，因为多肽的部分包含与在宿主细胞中正常表达的多肽相同或相似的氨基酸序列，而其它部分对于宿主细胞是外来的。

[0079] 替代酶：如本文中所用，术语“替代酶”是指可用于至少部分地替代待治疗的疾病中的有缺陷或缺失的酶的任何酶。在一些实施方案中，术语“替代酶”是指可用于至少部分地替代待治疗的溶酶体贮积症中的有缺陷或缺失的溶酶体酶的任何酶。在一些实施方案中，替代酶能够减少哺乳动物溶酶体中积累的材料或者能够挽救或改善一种或多种溶酶体

贮积病症状。适合于本发明的替代酶包括野生型溶酶体酶或经修饰的溶酶体酶并且可使用重组的和合成的方法来产生或从自然来源纯化而来。替代酶可以是重组的、合成的、基因活化的或天然的酶。

[0080] 接种：如本文中所用，术语“接种”是指给生物反应器或另一个器皿提供细胞培养物以进行大规模细胞培养生产的过程。在一些实施方案中，使用“种子培养物”，其中在接种之前，已在更小的细胞培养皿即组织培养瓶、组织培养平板、组织培养滚瓶等中对细胞进行了增殖。或者，在一些实施方案中，细胞可能已被冷冻，并且解冻后立即将它们提供给生物反应器或器皿。该术语是指任何数目的细胞，包括单个细胞。

[0081] 受试者：如本文中所用，术语“受试者”意指任何哺乳动物，包括人。在本发明的某些实施方案中，受试者是成人、青少年或婴儿。本发明还涵盖的是药物组合物的施用和/或子宫内治疗的方法的性能。

[0082] 滴度：如本文中所用，术语“滴度”是指通过细胞培养产生的重组表达的肽或蛋白质的总量除以给定量的培养基体积。

[0083] 载体：如本文中所用，“载体”指能够运输与其缔合的另一核酸的核酸分子。在一些实施例中，载体能够在宿主细胞例如真核和/或原核细胞中染色体外复制和/或表达与其连接的核酸。能够指导可操作地连接的基因的表达的载体在本文中称为“表达载体”。

[0084] 活细胞密度：如本文中所用，术语“活细胞密度”是指每单位体积的活细胞的数目。

[0085] 发明详述

[0086] 本发明除其它事项以外提供了用于在无血清培养基中使用哺乳动物细胞的悬浮培养大规模生产重组 I2S 蛋白的方法和组合物。具体地，本发明使用共表达重组 I2S 蛋白和甲酰甘氨酸生成酶 (FGE) 的哺乳动物细胞。

[0087] 在以下小节中进一步详细地描述了本发明的各个方面。小节的使用并不意味着限制本发明。每一个小节可适用于本发明的任何方面。在本申请中，除非另有说明，否则“或”的使用表示“和/或”。

[0088] 艾杜糖 -2- 硫酸酯酶 (I2S)

[0089] 如本文中所用，I2S 蛋白是可取代天然存在的艾杜糖 -2- 硫酸酯酶 (I2S) 蛋白的至少部分活性或挽救一个或多个与 I2S 缺乏相关的表型或症状的任何蛋白质或蛋白质的部分。如本文中所用，术语“I2S 酶”和“I2S 蛋白”以及语法等同物可互换使用。

[0090] 通常情况下，人 I2S 蛋白作为前体形式产生。人 I2S 的前体形式包含信号肽（全长前体的氨基酸残基 1-25）、原肽（全长前体的氨基酸残基 26-33）和一条链（全长前体的残基 34-550），所述链可被进一步加工成 42kDa 的链（全长前体的残基的 34-455）和 14kDa 的链（全长前体的残基 446-550）。通常情况下，前体形式也称为全长前体或全长 I2S 蛋白，其含有 550 个氨基酸。已除去信号肽的成熟形式的氨基酸序列 (SEQ ID NO：1) 和典型野生型或天然存在的人 I2S 蛋白的全长前体的氨基酸序列 (SEQ ID NO:2) 示于表 1 中。信号肽加以下划线。此外，人 I2S 蛋白同种型 a 和 b 前体的氨基酸序列也分别提供于表 1 中的 SEQ ID NO：3 和 4。

[0091] 表 1. 人艾杜糖 -2- 硫酸酯酶

[0092]

成熟形式	SETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFA QQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSV GKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVD VLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKL YPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRK IRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYS NFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVEL VSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNP RELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFL ANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP(SEQ ID NO:1)
全长前体 (同种型a)	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCY GDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSY WRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSS EKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSA SPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDI RQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLA NSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEKLF PYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELC REGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIK

[0093]

	IMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQ GGDLFQLLMP(SEQ ID NO:2)
同种型b前体	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCY GDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSY WRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSS EKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSA SPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDI RQREDVQALNISVPYGPIPVDFQEDQSSTGFRLKTSSTRKYK (SEQ ID NO:3)
同种型c前体	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCY GDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSY WRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSS EKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSA SPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDI RQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLA NSTIIAFTSDHGFLMRTNT(SEQ ID No:4)

[0094] 因此,在一些实施方案中,I2S 酶为成熟人 I2S 蛋白 (SEQ ID NO:1)。如本文中公开的,SEQ ID NO:1 代表人 I2S 蛋白的典型氨基酸序列。在一些实施方案中,I2S 蛋白可以是因在 I2S 基因的 5’UTR 内的可选择起始位点上的转录产生的 SEQ ID NO:1 的剪接同种型和 / 或变体。在一些实施方案中,适合的替代酶可以是成熟人 I2S 蛋白的同源物或类似物。例如,成熟人 I2S 蛋白的同源物或类似物可以是相较于野生型或天然存在的 I2S 蛋白 (例如, SEQ ID NO:1) 包含一个或多个氨基酸取代、缺失和 / 或插入,同时保留大部分 I2S 蛋白活性的修饰成熟人 I2S 蛋白。因此,在一些实施方案中,适合于本发明的替代酶与成熟人 I2S 蛋白 (SEQ ID NO:1) 大体上同源。在一些实施方案中,适合于本发明的替代酶具有与 SEQ ID NO:1 具有至少 50%、55%、60%、65%、70%、75%、80%、85%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%或更大的同源性的氨基酸序列。在一些实施方案中,适合于本发明的替代酶与成熟人 I2S 蛋白 (SEQ ID NO:1) 大体上同一。在一些实施方案中,适合于本发明的替代酶具有与 SEQ ID NO:1 具有至少 50%、55%、60%、65%、70%、75%、80%、85%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%或更大同一性的氨基酸序列。在一些实施方案中,适合于本发明的替代酶包含成熟人 I2S 蛋白的片段或部分。

[0095] 或者，I2S 酶是全长 I2S 蛋白。在一些实施方案中，I2S 酶可以是全长人 I2S 蛋白的同源物或类似物。例如，全长人 I2S 蛋白的同源物或类似物可以是相较于野生型或天然存在的全长 I2S 蛋白（例如，SEQ ID NO:2）包含一个或多个氨基酸取代、缺失和/或插入，同时保留大部分 I2S 蛋白活性的修饰全长人 I2S 蛋白。因此，在一些实施方案中，I2S 酶与全长人 I2S 蛋白（SEQ ID NO:2）大体上同源。在一些实施方案中，适合于本发明的 I2S 酶具有与 SEQ ID NO:2 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更大同源性的氨基酸序列。在一些实施方案中，适合于本发明的 I2S 酶与 SEQ ID NO:2 大体上同一。在一些实施方案中，适合于本发明的 I2S 酶具有与 SEQ ID NO:2 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同一性的氨基酸序列。在一些实施方案中，适合于本发明的 I2S 酶包含全长人 I2S 蛋白的片段或部分。如本文中所用，全长 I2S 蛋白通常包含信号肽序列。

[0096] 在一些实施方案中，适合于本发明的 I2S 酶为人 I2S 同种型 a 蛋白。在一些实施方案中，适合的 I2S 酶可以是人 I2S 同种型 a 蛋白的同源物或类似物。例如，人 I2S 同种型 a 蛋白的同源物或类似物可以是相较于野生型或天然存在的人 I2S 同种型 a 蛋白（例如，SEQ ID NO:3）包含一个或多个氨基酸取代、缺失和/或插入，同时保留大部分 I2S 蛋白活性的修饰人 I2S 同种型 a 蛋白。因此，在一些实施方案中，I2S 酶与人 I2S 同种型 a 蛋白（SEQ ID NO:3）大体上同源。在一些实施方案中，I2S 酶具有与 SEQ ID NO:3 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同源性的氨基酸序列。在一些实施方案中，I2S 酶与 SEQ ID NO:3 大体上同一。在一些实施方案中，适用于本发明的 I2S 酶具有与 SEQ ID NO:3 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同一性的氨基酸序列。在一些实施方案中，适用于本发明的 I2S 酶包含人 I2S 同种型 a 蛋白的片段或部分。如本文中所用，人 I2S 同种型 a 蛋白通常包含信号肽序列。

[0097] 在一些实施方案中，I2S 酶为人 I2S 同种型 b 蛋白。在一些实施方案中，I2S 酶可以是人 I2S 同种型 b 蛋白的同源物或类似物。例如，人 I2S 同种型 b 蛋白的同源物或类似物可以是相较于野生型或天然存在的人 I2S 同种型 b 蛋白（例如，SEQ ID NO:4）包含一个或多个氨基酸取代、缺失和/或插入，同时保留大部分 I2S 蛋白活性的修饰人 I2S 同种型 b 蛋白。因此，在一些实施方案中，I2S 酶与人 I2S 同种型 b 蛋白（SEQ ID NO:4）大体上同源。在一些实施方案中，I2S 酶具有与 SEQ ID NO:4 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同源性的氨基酸序列。在一些实施方案中，I2S 酶与 SEQ ID NO:4 大体上同一。在一些实施方案中，I2S 酶具有与 SEQ ID NO:4 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同一性的氨基酸序列。在一些实施方案中，适合于本发明的 I2S 酶包含人 I2S 同种型 b 蛋白的片段或部分。如本文中所用，人 I2S 同种型 b 蛋白通常包含信号肽序列。

[0098] 人 I2S 蛋白的同源物或类似物可按照对于本领域普通技术人员来说是已知的用于改变多肽序列的方法，例如见于汇编此类方法的参考资料中的方法来制备。在一些实施方案中，氨基酸的保守取代包括在下列组内的氨基酸间的置换：(a)M、I、L、V；(b)F、Y、W；

(c)K、R、H；(d)A、G；(e)S、T；(f)Q、N；和 (g)E、D。在一些实施方案中，“保守氨基酸取代”是指不改变在其中进行氨基酸取代的蛋白质的相关电荷或大小特征的氨基酸取代。

[0099] 在一些实施方案中，I2S 酶包含结合于细胞表面上的受体以促进细胞摄取和 / 或溶酶体靶向的部分。例如，这样的受体可以是不依赖于阳离子的甘露糖 -6- 磷酸受体 (CI-MPR)，其结合甘露糖 -6- 磷酸 (M6P) 残基。此外，CI-MPR 也结合其它蛋白质，包括 IGF- Ⅱ。适合的溶酶体靶向部分可以是 IGF-I、IGF-II、RAP、p97 及其变体、同源物或片段 (例如，包括那些具有与野生型成熟人 IGF-I、IGF-II、RAP、p97 肽序列具有至少 70％、75％、80％、85％、90％或 95％同一性的序列的肽)。在一些实施方案中，M6P 残基结合的受体可以是阳离子依赖性的。

[0100] 甲酰甘氨酸生成酶 (FGE)

[0101] 通常情况下，I2S 的酶活性受保守半胱氨酸 (例如，对应于成熟人 I2S(SEQ ID NO:1) 的氨基酸 59) 至甲酰甘氨酸的翻译后修饰影响，所述甲酰甘氨酸也被称为 2- 氨基 -3- 氧代丙酸或氧代 - 丙氨酸。这种翻译后修饰一般在蛋白质合成过程中发生于内质网中，并且由甲酰甘氨酸生成酶 (FGE) 催化。I2S 的酶比活性通常与 I2S 具有甲酰甘氨酸修饰所达到的程度正相关。例如，具有相对高的甲酰甘氨酸修饰量的 I2S 蛋白制剂通常具有相对高的酶比活性；然而具有相对低的甲酰甘氨酸修饰量的 I2S 蛋白制剂通常具有相对低的酶比活性。

[0102] 因此，适合于产生根据本发明的重组 I2S 蛋白的细胞通常表达 FGE 蛋白。在一些实施方案中，适合的细胞表达内源性蛋白 FGE。在一些实施方案中，适合的细胞经工程化以表达与重组 I2S 组合的外源或重组的甲酰甘氨酸生成酶 (FGE)。在一些实施方案中，适合的细胞经改造以激活内源 FGE 基因以便 FGE 蛋白的表达水平或活性升高。

[0103] 通常情况下，人 FGE 蛋白产生为前体形式。人 FGE 的前体形式包含信号肽 (全长前体的氨基酸残基 1-33) 和链 (全长前体的残基 34-374)。通常情况下，前体形式也称为为全长前体或全长 FGE 蛋白，其含有 374 个氨基酸。除去信号肽的成熟形式的氨基酸序列 (SEQ IDNO：5) 和典型野生型或天然存在的人 FGE 蛋白的全长前体的氨基酸序列 (SEQ ID NO:6) 示于表 2 中。

[0104] 表 2. 人甲酰甘氨酸生成酶 (FGE)

[0105]

成熟形式	SQEAGTGAGAGSLAGSCGCGTPQRPGAHGSSAAAHRYSREANAPGPVPGERQLA HSKMVPIPAGVFTMGTDDPQIKQDGEAPARRVTIDAFYMDAYEVSNTEFEKFVN STGYLTEAEKFGDSFVFEGMLSEQVKTNIQQAVAAAPWWLPVKGANWRHPEGPD STILHRPDHPVLHVSWNDAVAYCTWAGKRLPTEAEWEYSCRGGLHNRLFPWGNK LQPKGQHYANIWQGEFPVTNTGEDGFQGTAPVDAFPPNGYGLYNIVGNAWEWTS DWWTVHHSVEETLNPKGPPSGKDRVKKGGSYMCHRSYCYRYRCAARSQNTPDSS ASNLGFRCAADRLPTMD (SEQ ID NO:5)
全长前体	MAAPALGLVCGRCPELGLVLLLLLLSLLCGAAGSQEAGTGAGAGSLAGSCGCGT PQRPGAHGSSAAAHRYSREANAPGPVPGERQLAHSKMVPIPAGVFTMGTDDPQI KQDGEAPARRVTIDAFYMDAYEVSNTEFEKFVNSTGYLTEAEKFGDSFVFEGML SEQVKTNIQQAVAAAPWWLPVKGANWRHPEGPDSTILHRPDHPVLHVSWNDAVA YCTWAGKRLPTEAEWEYSCRGGLHNRLFPWGNKLQPKGQHYANIWQGEFPVTNT GEDGFQGTAPVDAFPPNGYGLYNIVGNAWEWTSDWWTVHHSVEETLNPKGPPSG KDRVKKGGSYMCHRSYCYRYRCAARSQNTPDSSASNLGFRCAADRLPTMD (SEQ ID NO:6)

[0106] 因此，在一些实施方案中，适合本发明的 FGE 酶为成熟人 FGE 蛋白 (SEQ ID NO:5)。

在一些实施方案中，适合的 FGE 酶可以是成熟人 FGE 蛋白的同源物或类似物。例如，成熟人 FGE 蛋白的同源物或类似物可以是相较于野生型或天然存在的 FGE 蛋白（例如，SEQ ID NO:5）包含一个或多个氨基酸取代、缺失和 / 或插入，同时保留大部分 FGE 蛋白活性的修饰成熟人 FGE 蛋白。因此，在一些实施方案中，适合于本发明的 FGE 酶与成熟人 FGE 蛋白（SEQ ID NO:5）大体上同源。在一些实施方案中，适合于本发明的 FGE 酶具有与 SEQ ID NO:5 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同源性的氨基酸序列。在一些实施方案中，适合于本发明的 FGE 酶与成熟人 FGE 蛋白（SEQ ID NO:5）大体上同一。在一些实施方案中，适合于本发明的 FGE 酶具有与 SEQ ID NO:5 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同一性的氨基酸序列。在一些实施方案中，适合于本发明的 FGE 酶包含成熟人 FGE 蛋白的片段或部分。

[0107] 或者，适合本发明的 FGE 酶为全长 FGE 蛋白。在一些实施方案中，FGE 酶可以是全长人 FGE 蛋白的同源物或类似物。例如，全长人 FGE 蛋白的同源物或类似物可以是相较于野生型或天然存在的全长 FGE 蛋白（例如，SEQ ID NO:6）包含一个或多个氨基酸取代、缺失和 / 或插入，同时保留大部分 FGE 蛋白活性的修饰全长人 FGE 蛋白。因此，在一些实施方案中，适合于本发明的 FGE 酶与全长人 FGE 蛋白（SEQ ID NO:6）大体上同源。在一些实施方案中，适合于本发明的 FGE 酶具有与 SEQ ID NO:4 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同源性的氨基酸序列。在一些实施方案中，适合于本发明的 FGE 酶与 SEQ ID NO:6 大体上同一。在一些实施方案中，适合于本发明的 FGE 酶具有与 SEQ ID NO:6 具有至少 50％、55％、60％、65％、70％、75％、80％、85％、90％、91％、92％、93％、94％、95％、96％、97％、98％、99％或更高同一性的氨基酸序列。在一些实施方案中，适合于本发明的 FGE 酶包含全长人 FGE 蛋白的片段或部分。如本文中所用，全长 FGE 蛋白通常包含信号肽序列。

[0108] 编码示例性 FGE 蛋白的示例性核酸序列和氨基酸序列公开于 US 公开号 20040229250 中，其全部内容通过引用并入本文。

[0109] 宿主细胞

[0110] 如本文中所用，术语“宿主细胞”指可用于产生重组 I2S 酶的细胞。具体地，宿主细胞适合于大规模产生重组 I2S 酶。在一些实施方案中，宿主细胞能够以约 5 皮克 / 细胞 / 日的量或大于 5 皮克 / 细胞 / 日的量（例如，大于约 10、15、20、25、30、35、40、45、50、55、60、65、70、75、80、85、90、95 或 100 皮克 / 细胞 / 日）产生 I2S 酶。在一些实施方案中，宿主细胞能够以在约 5-100 皮克 / 细胞 / 日（例如，约 5-90 皮克 / 细胞 / 日、约 5-80 皮克 / 细胞 / 日、约 5-70 皮克 / 细胞 / 日、约 5-60 皮克 / 细胞 / 日、约 5-50 皮克 / 细胞 / 日、约 5-40 皮克 / 细胞 / 日、约 5-30 皮克 / 细胞 / 日、约 10-90 皮克 / 细胞 / 日、约 10-80 皮克 / 细胞 / 日、约 10-70 皮克 / 细胞 / 日、约 10-60 皮克 / 细胞 / 日、约 10-50 皮克 / 细胞 / 日、约 10-40 皮克 / 细胞 / 日、约 10-30 皮克 / 细胞 / 日、约 20-90 皮克 / 细胞 / 日、约 20-80 皮克 / 细胞 / 日、约 20-70 皮克 / 细胞 / 日、约 20-60 皮克 / 细胞 / 日、约 20-50 皮克 / 细胞 / 日、约 20-40 皮克 / 细胞 / 日、约 20-30 皮克 / 细胞 / 日）的范围内的量产生 I2S 酶。在一些实施方案中，适合的宿主细胞不是内体酸化缺陷型细胞。

[0111] 适合的宿主细胞可来源于多种生物体，包括但不限于哺乳动物、植物、鸟类（例如，

禽系统）、昆虫、酵母和细菌。在一些实施方案中，宿主细胞是哺乳动物细胞。可按照本发明将易于进行细胞培养和多肽表达的任何哺乳动物细胞用作宿主细胞。可按照本发明使用的哺乳动物细胞的非限制性实例包括人胚胎肾 293 细胞 (HEK293)、HeLa 细胞、BALB/c 小鼠骨髓瘤细胞系 (NSO/1，ECACC 号 :85110503)、人成视网膜细胞 (PER.C6(CruCell,Leiden,The Netherlands))、由 SV40 转化的猴肾 CV1 细胞系 (COS-7，ATCC CRL 1651)、人成纤维肉瘤细胞系（例如，HT-1080)、人胚肾系（经亚克隆以在悬浮培养物中生长的 293 或 293 细胞，Graham 等人，J.Gen Virol.,36:59(1977))、幼仓鼠肾细胞 (BHK，ATCC CCL10)、中国仓鼠卵巢细胞 +/-DHFR(CHO,Urlaub 和 Chasin,Proc.Natl.Acad.Sci.USA,77:4216(1980))、小鼠支持细胞 (sertoli cell)(TM4,Mather,Biol.Reprod.,23:243-251(1980))、猴肾细胞 (CV1ATCC CCL70)、非洲绿猴肾细胞 (VERO-76，ATCC CRL-1587)、人宫颈癌细胞 (HeLa，ATCC CCL 2)、犬肾细胞 (MDCK,ATCC CCL34)、buffalo 大鼠肝细胞 (BRL3A,ATCC CRL 1442)、人肺细胞 (W138,ATCC CCL 75)、人肝细胞病毒 (Hep G2,HB 8065)、小鼠乳腺肿瘤 (MMT 060562，ATCC CCL51)、TRI 细胞 (Mather 等人，Annals N.Y.Acad.Sci.,383:44-68(1982))、MRC 5 细胞、FS4 细胞、人肝细胞瘤细胞系 (Hep G2)、人细胞系 CAP 和 AGE1.HN. 和 Glycotope'小组。

[0112] 此外，可按照本发明使用任何数目的可得的杂交瘤细胞系。本领域技术人员将理解，杂交瘤细胞系可能具有不同的营养需要和 / 或可能需要不同的培养条件来进行最佳生长和多肽或蛋白质表达，并将能够根据需要来修改条件。

[0113] 在一些实施方案中，宿主细胞是非哺乳动物细胞。适合于本发明的非哺乳动物宿主细胞的非限制性实例包括来源于巴斯德毕赤酵母 (Pichia pastoris)、甲醇毕赤酵母 (Pichia methanolica)、安格斯毕赤酵母 (Pichia angusta)、粟酒裂殖酵母 (Schizosacccharomyces pombe)、酿酒酵母 (Saccharomyces cerevisiae) 和解脂耶氏酵母 (Yarrowia lipolytica)（对于酵母）；草地夜蛾 (Sodoptera frugiperda)、粉纹夜蛾 (Trichoplusis ni)、黑腹果蝇 (Drosophila melangoster) 和烟草天蛾 (Manduca sexta)（对于昆虫）；和大肠杆菌 (Escherichia coli)、鼠伤寒沙门氏菌 (Salmonella typhimurium)、枯草芽孢杆菌 (Bacillus subtilis)、地衣形芽孢杆菌 (Bacillus lichenifonnis)、脆弱拟杆菌 (Bacteroides fragilis)、产气荚膜梭状芽胞杆菌 (Clostridia perfringens)、难辨梭状芽孢杆菌 (Clostridia difficile)（对于细菌）；和来自两栖动物的非洲爪蟾 (Xenopus Laevis) 的细胞和细胞系。

[0114] 载体和核酸构建体

[0115] 可将各种核酸构建体用于在宿主细胞中表达本文中描述的 I2S 和 / 或 FGE 酶。适合的载体构建体，除了 I2S 和 / 或 FGE 蛋白编码序列（也称为 I2S 或 FGE 转基因）以外，通常还包含调控序列、基因控制序列、启动子、非编码序列和 / 或用于蛋白质表达和任选地构建体复制的其它适当的序列。通常情况下，将编码区与这些核酸组件中的一个或多个可操作地连接。

[0116] “调控序列”通常是指位于编码序列的上游 (5' 非编码序列）、内部或下游 (3' 非编码序列）并且影响连接的编码序列的转录、RNA 加工或稳定性或翻译的核苷酸序列。调控序列可包括启动子、增强子、5' 非翻译序列、翻译前导序列、内含子和 3' 非翻译序列例如聚腺苷酸化识别序列。有时，“调控序列”也称为“基因控制序列”。

[0117] “启动子”通常是指能够控制编码序列或功能性 RNA 的表达的核苷酸序列。一般

地，编码序列位于启动子序列的3’。启动子序列由近端和更远端上游元件组成，后一元件通常称为增强子。相应地，“增强子”为可刺激启动子活性，并且可以为启动子的固有元件或被插入以增强启动子的水平或组织特异性的异源元件的核苷酸序列。启动子可整体上来源于天然基因或由来源于天然发现的不同启动子的不同元件组成或甚至包含合成核苷酸区段。本领域普通技术人员应理解，不同的启动子可在不同组织或细胞类型中或在不同的发育阶段或响应于不同的环境条件来指导基因的表达。

[0118] “3’非编码序列”通常是指位于编码序列下游的核苷酸序列，并且包括聚腺苷酸化识别序列和编码能够影响 mRNA 加工或基因表达的调控信号的其它序列。聚腺苷酸化信号的特征通常在于影响聚腺苷酸段至 mRNA 前体的 3’末端的添加。

[0119] “翻译前导序列”或“5’非编码序列”通常是指位于基因的启动子序列与编码序列之间的核苷酸序列。翻译前导序列存在于翻译起始序列上游的完全加工的 mRNA 中。翻译前导序列可影响初级转录物至 mRNA 的加工、mRNA 稳定性或翻译效率。

[0120] 通常情况下，术语“可操作地连接的”是指两个或更多个核酸片段在单个核酸片段上的连接，以便一个核酸片段的功能受另一个核酸片段的影响。例如，当启动子能够影响该编码序列的表达（即，编码序列处于启动子的转录控制之下）时，所述启动子与所述编码序列可操作地连接。编码序列可以以有义或反义定向可操作地连接于调控序列。

[0121] 转基因的编码区可包含一个或多个沉默突变来优化用于特定细胞类型的密码子选择。例如，可优化 I2S 转基因的密码子以用于在脊椎动物细胞中表达。在一些实施方案中，可优化 I2S 转基因的密码子以用于在哺乳动物细胞中表达。在一些实施方案中，可优化 I2S 转基因的密码子以用于在人细胞中表达。

[0122] 任选地，构建体可包含另外的组件例如下列组件的一个或多个：剪接位点、增强子序列、在适当的启动子控制下的可选择标记基因、在适当的启动子控制下的可扩增标志基因和基质附着区 (MAR) 或本领域中已知的其它元件，所述元件增强其所被插入的区域的表达。

[0123] 一旦转染或转导入宿主细胞后，适合的载体可在染色体外（附加型地）表达或整合进入宿主细胞的基因组。

[0124] 在一些实施方案中，整合入细胞的基因组的 DNA 构建体，其需要仅包括转基因核酸序列。在该情况下，通常通过整合位点上的调控序列来控制转基因的表达。任选地，其可包括本文中描述的另外的各种调控序列。

[0125] 培养基

[0126] 如本文中所用，术语“培养基”和“培养介质”是指一般种类的包含适合于体外维持和 / 或生长细胞的营养物质的溶液。通常情况下，培养基溶液提供，但不限于，细胞进行至少最低生长和 / 或存活所需的必需和非必需氨基酸、维生素、能量来源、脂质和微量元素。在其它实施方案中，培养基可包含来源于本领域已知的任何来源或方法的氨基酸，包括但不限于，来源于单个氨基酸添加或来源于蛋白胨或蛋白质水解产物添加（包括动物或植物来源）的氨基酸。维生素例如但不限于，生物素、泛酸盐、氯化胆碱、叶酸、肌醇、烟酰胺、吡哆醇、核黄素、维生素 B12、硫胺素、腐胺和 / 或其组合。盐例如但不限于，$CaCl_2$、KCl、$MgCl_2$、NaCl、磷酸二氢钠、磷酸氢二钠、亚硒酸钠、$CuSO_4$、$ZnCl_2$及其组合。脂肪酸例如但不限于，花生四烯酸、亚油酸、油酸、月桂酸、肉豆蔻酸以及甲基 - β - 环糊精和 / 或其组合）。在一些实

施方案中，培养基包含额外组分如葡萄糖、谷氨酰胺、丙酮酸钠、胰岛素或乙醇胺、保护剂例如Pluronic F68。在一些实施方案中，培养基还可包含使生长和/或存活增强，高于最小速率的组分，包括激素和生长因子。培养基还可包含一种或多种缓冲剂。缓冲剂可被设计和/或选择来在特定pH(例如，生理pH值，(例如，pH 6.8至pH 7.4))下维持培养物。多种适合用于培养细胞的缓冲剂在本领域中是已知的并且可用于所述方法。适合的缓冲剂(例如，碳酸氢盐缓冲剂、HEPES缓冲剂、Good氏缓冲剂等)是指那些具有维持生理pH(尽管存在与细胞呼吸相关的二氧化碳浓度的变化)的能力和效率的缓冲剂。优选将溶液配制成对于细胞存活和增殖是最佳的pH和盐浓度。

[0127] 在一些实施方案中，培养基可以是化学成分确定的培养基。如本文中所用，术语“化学成分确定的营养培养基”是指大体上全部化学成分都是已知的培养基。在一些实施方案中，化学成分确定的营养培养基不含动物来源的组分。在一些情况下，化学成分确定的培养基包含一种或多种蛋白质(例如，蛋白生长因子或细胞因子)。在一些情况下，化学成分确定的营养培养基包含一种或多种蛋白质水解产物。在其它情况下，化学成分确定的营养培养基为不含蛋白质的培养基，即不含蛋白质、水解产物或未知组成的组分的无血清培养基。

[0128] 通常情况下，通过以预定的重量或摩尔百分比或比率组合各种单独的组分例如必需和非必需氨基酸、维生素、能量来源、脂质、盐、缓冲剂和微量元素来制备化学成分确定的培养基。示例性的无血清培养基，特别地化学成分确定的培养基描述于美国公开号2006/0148074中，其公开内容在此通过引用并入。

[0129] 在一些实施方案中，适合于本发明的化学成分确定的培养基是商购可得的培养基，例如但不限于达尔伯克氏改良伊格尔氏培养基(Dulbecco's Modified Eagle's Medium,DMEM)、DMEM F12(1:1)、Ham营养混合物F-10、洛斯维帕克纪念研究所培养基(Roswell Park Memorial Institute Medium,RPMI)、MCDB 131、William培养基E、CD CHO培养基(Invitrogen®)、CD 293培养基(Invitrogen®)、EX-Cell CDCHO、Ex-Cell CDCHO Fusion、CD-OptiCHO、CD-FortiCHO、CDM4CHO、CD1000、BalanCD-CHO、IS-CHO-CD、CD杂交瘤、CD-DG44。在一些实施方案中，适合于本发明的化学成分确定的培养基为一种或多种商购可得化学成分确定的培养基的混合物。在各个实施方案中，适合的培养基为2、3、4、5、6、7、8、9、10或更多种商购可得化学成分确定的培养基的混合物。在一些实施方案中，每一种单独的商购可得化学成分确定的培养基(例如，如本文中描述的那些培养基)按重量计构成混合物的1%、2.5%、5%、7.5%、10%、12.5%、15%、20%、25%、30%、35%、40%、45%、50%、55%、60%、65%、70%、75%、80%、85%、90%、95%或更多。每一种单独成分培养基之间的比率可通过存在于混合物中的相对重量百分比来确定。

[0130] 在一些实施方案中，化学成分确定的培养基可补充以一种或多种动物来源的组分。此类动物来源的组分包括但不限于胎牛血清、马血清、山羊血清、驴血清、人血清和血清来源的蛋白质例如白蛋白(例如，牛血清白蛋白或人血清白蛋白)。

[0131] 氧化还原调节剂

[0132] 在一些实施方案中，适合的培养基包含一种或多种氧化还原调节剂。不希望受特定理论束缚，预期氧化还原调节剂可提高I2S的产量和/或活性，从而产生具有高水平的活性酶的重组I2S组合物。如本文中所用，“氧化还原调节剂”为影响混合物中的组分将获

得电子并从而被还原的可能性的分子（例如，小分子、多肽等）。氧化还原调节剂可增加或减小混合物中的组分将获得电子并从而被还原的可能性。在一些实施方案中，氧化还原调节剂可以已存在于培养基中，例如，当从商购可得来源获得化学成分确定的培养基或可作为添加剂提供给培养基时。在一些情况下，根据本发明的培养基包含两种或更多种氧化还原调节剂。氧化还原调节剂的非限制性实例包括谷胱甘肽、葡萄糖 -6- 磷酸、肌肽、鼠尾草酚、萝卜硫素、生育酚、抗坏血酸、脱氢抗坏血酸、硒、2- 巯基乙醇、N- 乙酰半胱氨酸、半胱氨酸、核黄素、烟酸、叶酸、黄素腺嘌呤二核苷酸 (FAD)、二硫苏糖和烟酰胺腺嘌呤二核苷酸磷酸 (NADP)。其它适当的氧化还原调节剂对于本领域普通技术人员来说是显而易见的。

[0133] 在一些实施方案中，将半胱氨添加至本发明的培养基中，或其存在于所述培养基中。半胱氨酸可以以不同的浓度存在。在一些实施方案中，培养基中半胱氨酸的浓度在约 0.1mg/L 至约 10mg/L、约 1mg/L 至约 25mg/L、约 10mg/L 至约 50mg/L、约 25mg/L 至约 65mg/L、约 10mg/L 至约 100mg/L 或约 25mg/L 至约 250mg/L 的范围内。在一些实施方案中，半胱氨酸的浓度的在约 0.1mg/L 至约 65mg/L（例如，1-50mg/L、1-40mg/L、1-30mg/l、1-20mg/L、1-10mg/L）的范围内。在一些情况下，培养基中半胱氨酸的浓度高达约 0.1mg/L、约 1mg/L、约 5mg/L、约 10mg/L、约 20mg/L、约 25mg/L、约 50mg/L、约 65mg/L、约 75mg/L、约 100mg/L 或更高。

[0134] 在一些实施方案中，将 2- 巯基乙醇添加至本发明的培养基中，或其存在于所述培养基中。可使用各种浓度。在一些实施方案中，2- 巯基乙醇的浓度在约 0.1nM 至约 0.001mM、约 0.001mM 至约 0.01mM、约 0.001mM 至约 0.1mM、约 0.01mM 至约 0.1mM、约 0.01mM 至约 1mM 的范围内。在一些情况下，2- 巯基乙醇的浓度高达约 0.1nM、约 0.001mM、约 0.01mM、约 0.1mM、约 1mM 或或更高。在一些实施方案中，2- 巯基乙醇的浓度在约 0.001mM 至约 0.01mM（例如，约 0.001-0.008mM、约 0.001-0.007mM、约 0.001-0.006mM、约 0.001-0.005mM、约 0.001-0.004mM、约 0.001-0.003mM、约 0.001-0.002mM）的范围内。

[0135] 在一些实施方案中，将 N- 乙酰半胱氨酸添加至本发明的培养基中，或其存在于所述培养基中。可使用各种浓度。在一些实施方案中，N- 乙酰半胱氨酸的浓度可在约 0.1mM 至约 1mM、约 1mM 至约 10mM、约 3mM 至约 9mM、约 1mM 至约 50mM 或约 10mM 至约 50mM 的范围内。在一些实施方案中，N- 乙酰半胱氨酸的浓度在约 3mM 至约 9mM（例如，约 3-8mM、约 3-7mM、约 3-6mM、约 3-5mM、约 3-4mM）的范围内。在一些实施方案中，N- 乙酰半胱氨酸的浓度可高达约 0.1mM、约 1mM、约 3mM、约 9mM、约 10mM、约 20mM、约 30mM、约 40mM、约 50mM 或更高。

[0136] 生长调节剂

[0137] 在一些实施方案中，培养基可包含一种或多种生长调节剂来提高 I2S 的产量。如本文中所用，术语“生长调节剂”是指影响细胞的生长的分子。生长调节剂可通过例如增强或诱导细胞增殖、细胞周期进展来增强细胞生长，或通过例如促进细胞周期停滞来减弱细胞生长。尽管商购可得培养基通常包含多种不同的生长调节剂，但在一些情况下期望向营养培养基提供额外的生长调节剂。因此，在一些实施方案中，向培养基添加一种或多种生长调节剂。

[0138] 在一些情况下，适合于本发明的生长调节剂包括次黄嘌呤。在一些实施方案中，次黄嘌呤的浓度在约 0.01mM 至约 0.1mM、约 0.1mM 至约 1mM、约 0.1mM 至约 10mM、约 1mM 至约 10mM、约 0.1mM 至约 100mM 的范围内。在一些实施方案中，次黄嘌呤的浓度在约 0.1mM 至

约 10mM（例如，约 0.1-9mM、约 0.1-8mM、约 0.1-7mM、约 0.1-6mM、约 0.1-5mM、约 0.1-4mM、约 0.1-3mM、约 0.1-2mM、约 0.1-1mM）的范围内。在一些情况下，次黄嘌呤的浓度为约 0.01mM、约 0.1mM、约 1mM、约 10mM、约 20mM、约 30mM、约 40mM、约 50mM、约 60mM、约 70mM、约 80mM、约 90mM、约 100mM 或更高。

[0139] 在一些情况下，适合于本发明的生长调节剂包括胸苷。在一些实施方案中，胸苷的浓度在约 0.01mM 至约 0.1mM、约 0.1mM 至约 1mM、约 0.1mM 至约 10mM、约 1mM 至约 10mM、约 0.1mM 至约 100mM、约 1mM 至约 100mM 的范围内。在一些实施方案中，胸苷的浓度在约 1mM 至约 100mM（例如，约 1-90mM、约 1-80mM、约 1-70mM、约 1-60mM、约 1-50mM、约 1-40mM、约 1-30mM、约 1-20mM、约 1-10mM）的范围内。在一些实施方案中，胸苷的浓度为约 0.01mM、约 0.1mM、约 1mM、约 10mM、约 20mM、约 30mM、约 40mM、约 50mM、约 60mM、约 70mM、约 80mM、约 90mM、约 100mM 或更高。

[0140] 培养条件

[0141] 本发明提供了大规模生产重组 I2S 的方法。用于产生目标重组多肽的常见大规模方法程序包括分批培养和补料分批培养。分批培养法常规地包括用特定细胞密度的种子培养物接种大规模生产培养物，在有益于细胞生长、活力和 / 或生产率的条件（例如，适合的培养基、pH 和温度）下使细胞生长，当细胞达到指定的细胞密度时收获细胞，和纯化表达的多肽。补料分批培养法包括用营养物和在细胞生长过程中被消耗的其它组分补充分批培养物的额外步骤。在一些实施方案中，根据本发明的大规模生产方法使用补料分批培养系统。

[0142] 培养起始

[0143] 通常情况下，首先通过本领域普通技术人员公知的多种方法中的任何一种增殖期望的表达 I2S 蛋白的细胞。通常通过使细胞在对细胞的存活、生长和活力有益的温度下和培养基中生长来增殖所述细胞。初始培养体积可以是任何大小，但通常小于用于最终生产的生产生物反应器的培养体积，并且在接种生产生物反应器之前，通常将细胞以递增的培养体积传代数次。可搅拌或摇动细胞培养物以增强培养基的充氧作用和营养物至细胞的扩散。或者或另外地，可将本领域公知的特殊喷射装置用于增强和控制培养物的充氧作用。

[0144] 可由本领域普通技术人员选择起始细胞密度。根据本发明，起始细胞密度可低至每培养体积单个细胞。在一些实施方案中，起始细胞密度可在约 $1X10^2$个活细胞 /mL 至约 $1X10^3$、$1X10^4$、$1X10^5$个活细胞 /mL 或更高的范围内。

[0145] 可使初始和中间细胞培养物生长至任何期望的密度，随后接种下一个中间或终生产生物反应器。在一些实施方案中，接种生产生物反应器之前的终活力大于约 70％、75％、80％、85％、90％、95％或更大。可以例如通过低速离心从上清液除去细胞。还可期望在接种下一个生物反应器之前用培养基洗涤取出的细胞以除去任何不想要的代谢废产物或培养基组分。培养基可以是先前使细胞在其中生长的培养基，或其可以是由本发明的实施者选择的不同的培养基或洗涤溶液。

[0146] 随后可将细胞稀释至适当的密度以接种生产生物反应器。在一些实施方案中，将细胞稀释至将用于生产生物反应器的相同培养基中。或者，可将细胞稀释至另一种培养基或溶液中，这取决于本发明的实施者的需要和期望，或适应细胞本身的特殊需要，例如，如果在接种生产生物反应器之前将它们短期贮存的话。

[0147] 生长期

[0148] 通常情况下，在已如上所述接种生产生物反应器后，在有益于细胞培养物的存活、生长和活力的条件下在初始生长期维持细胞培养物。根据本发明，生产生物反应器可以是适合用于蛋白质的大规模生产的任何体积。参见下面的“生物反应器”小节。

[0149] 主要基于细胞保持活力时所处的温度的范围来选择生长期中细胞培养的温度。可将生长期的温度维持在单一恒定的温度上或温度范围内。例如，在生长期期间中可稳定地升高或降低温度。一般地，大多数哺乳动物细胞在约 25℃至 42℃（例如，30℃至 40℃、约 30℃至 37℃、约 35℃至 40℃）的范围内生长良好。在一些实施方案中，于在约 30-37℃（例如，约 31-37℃、约 32-37℃、约 33-37℃、约 34-37℃、约 35-37℃、约 36-37℃）的范围内的温度下培养哺乳动物细胞。通常情况下，在生长期期间中，细胞在约 28℃、约 30℃、约 31℃、约 32℃、约 33℃、约 34℃、约 35℃、约 36℃、约 37℃、约 38℃、约 39℃、约 40℃下生长。

[0150] 可在初始生长期期间使细胞生长更多或更少量的时间，这取决于实施者的需要和细胞本身的要求。在一个实施方案中，使细胞生长持续足以获得为最大活细胞密度的给定百分比的活细胞密度的一段时间，如果使细胞不受干扰地生长，细胞最终可达到所述最大活细胞密度。例如，可使细胞生长持续足以获得为最大活细胞密度的 1％、5％、10％、15％、20％、25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％或 99％的期望活细胞密度的一段时间。

[0151] 在一些实施方案中，使细胞生长持续确定的一段时间。例如，取决于细胞培养物的起始浓度、细胞生长所处的温度以及细胞的固有生长速率，可使细胞生长 0、1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20 或更多日。在一些情况下，可使细胞生长一个月或更长时间。

[0152] 在一些实施方案中，使细胞生长至期望的活细胞密度。例如，生长期结束时期望的活细胞密度大于约 $1.0X10^6$个活细胞 /mL、$1.5X10^6$个活细胞 /mL、$2.0X10^6$个活细胞 /mL、$2.5X10^6$个活细胞 /mL、$5X10^6$个活细胞 /mL、$10X10^6$个活细胞 /mL、$20X10^6$个活细胞 /mL、$30X10^6$个活细胞 /mL、$40X10^6$个活细胞 /mL 或 $50X10^6$个活细胞 /mL。

[0153] 可在初始培养期期间搅拌或摇动细胞培养物，以增强充氧作用或营养物至细胞的扩散。根据本发明，本领域普通技术人员将理解，可有益地在初始生长期期间控制或调节生物反应器的某些内部条件，包括但不限于 pH、温度、充氧作用等。例如，pH 可通过提供适当量的酸或碱来控制，并且充氧作用可利用本领域公知的喷雾装置来控制。在一些实施方案中，用于生长期的期望的 pH 在约 6.8－7.5（例如，约 6.9-7.4、约 6.9-7.3、约 6.95-7.3、约 6.95-7.25、约 7.0-7.3、约 7.0-7.25、约 7.0-7.2、约 7.0-7.15、约 7.05-7.3、约 7.05-7.25、约 7.05-7.15、约 7.05-7.20、约 7.10-7.3、约 7.10-7.25、约 7.10-7.20、约 7.10-7.15）的范围内。在一些实施方案中，用于生长期的期望的 pH 为约 6.8、6.85、6.9、6.95、7.0、7.05、7.1、7.15、7.2、7.25、7.3、7.35、7.4、7.45 或 7.5。

[0154] 过渡期

[0155] 在一些实施方案中，当细胞准备用于生产期时，可改变培养条件以最大化目标重组蛋白的产量。这样的培养条件变化通常在过渡期中发生。在一些实施方案中，这样的变化可以是许多培养条件包括但不限于温度、pH、克分子渗透压浓度和培养基中的一个或多个的偏移。在一个实施方案中，使培养物的 pH 偏移。例如，从生长期至生产期可增加或减小培养基的 pH。在一些实施方案中，pH 的该变化是快速的。在一些实施方案中，pH 的该变

化在长时间中缓慢发生。在一些实施方案中，通过添加碳酸氢钠调节 pH 的变化。在一些实施方案中，在过渡期开始时起始 pH 的变化，并在随后的生产期期间维持该 pH。

[0156] 在一个实施方案中，使细胞培养基的葡萄糖浓度偏移。根据本实施方案，在过渡期开始后，将细胞培养中的葡萄糖浓度调整至高于 7.5mM 的等级。

[0157] 在一些实施方案中，从生长期至生产期将温度向上或向下偏移。例如，从生长期至生产期使温度向上或向下偏约 0.1℃、0.2℃、0.3℃、0.4℃、0.5℃、1.0℃、1.5℃、2.0℃、2.5℃、3.0℃、3.5℃、4.0℃、4.5℃、5.0℃或更多。

[0158] 生产期

[0159] 根据本发明，在细胞培养物达到期望的细胞密度和活力，经历或不经历过渡期后，在有益于细胞培养物的存活和活力并且适合于以商业上足够的水平表达 I2S 和 / 或 FGE 蛋白的条件下，维持细胞培养物进行随后的生产期。

[0160] 在一些实施方案中，在生产期期间，将培养物维持在低于生长期的温度或温度范围的温度或温度范围。例如，在生产期期间，细胞可在约 25℃至 35℃（例如，约 28℃至 35℃、约 30℃至 35℃、约 32℃至 35℃）的范围内良好地表达重组 I2S 和 / 或 FGE 蛋白。在一些实施方案中，在生产期期间，细胞可在约 25℃、约 26℃、约 27℃、约 28℃、约 29℃、约 30℃、约 31℃、约 32℃、约 33℃、约 34℃、约 35℃、约 36℃、约 37℃的温度下良好地表达重组 I2S 和 / 或 FGE 蛋白。在其它实施方案中，在生产期期间，将培养物维持在高于生长期的温度或温度范围的温度或温度范围。

[0161] 另外地或可选择地，在生产期期间，将培养物维持在与生长期的 pH 或 pH 范围不同（更低或更高）的 pH 或 pH 范围。在一些实施方案中，用于生产期的培养基具有在约 6.8-7.5（例如，约 6.9-7.4、约 6.9-7.3、约 6.95-7.3、约 6.95-7.25、约 7.0-7.3、约 7.0-7.25、约 7.0-7.2、约 7.0-7.15、约 7.05-7.3、约 7.05-7.25、约 7.05-7.15、约 7.05-7.20、约 7.10-7.3、约 7.10-7.25、约 7.10-7.20、约 7.10-7.15）的范围内的 pH。在一些实施方案中，培养基具有约 6.8、6.85、6.9、6.95、7.0、7.05、7.1、7.15、7.2、7.25、7.3、7.35、7.4、7.45 或 7.5 的 pH。

[0162] 在一些实施方案中，可在整个生产过程中将细胞维持在期望的细胞密度范围内。例如，在细胞培养物的生产期期间，期望的活细胞密度在生产期期间可在约 1.0-50X10^{6}个活细胞 /mL（例如，约 1.0-40X10^{6}个活细胞 /mL、约 1.0-30X10^{6}个活细胞 /mL、约 1.0-20X10^{6}个活细胞 /mL、约 1.0-10X10^{6}个活细胞 /mL、约 1.0-5X10^{6}个活细胞 /mL、约 1.0-4.5X10^{6}个活细胞 /mL、约 1.0-4X10^{6}个活细胞 /mL、约 1.0-3.5X10^{6}个活细胞 /mL、约 1.0-3X10^{6}个活细胞 /mL、约 1.0-2.5X10^{6}个活细胞 /mL、约 1.0-2.0X10^{6}个活细胞 /mL、约 1.0-1.5X10^{6}个活细胞 /mL、约 1.5-10X10^{6}个活细胞 /mL、约 1.5-5X10^{6}个活细胞 /mL、约 1.5-4.5X10^{6}个活细胞 /mL、约 1.5-4X10^{6}个活细胞 /mL、约 1.5-3.5X10^{6}个活细胞 /mL、约 1.5-3.0X10^{6}个活细胞 /mL、约 1.5-2.5X10^{6}个活细胞 /mL、约 1.5-2.0X10^{6}个活细胞 /mL）的范围内。

[0163] 在一些实施方案中，可维持细胞进行足以获得为最大活细胞密度的 1％、5％、10％、15％、20％、25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％或 99％的活细胞密度的时间。在一些情况下，可能期望使活细胞密度达到最大。在一些实施方案中，可能期望使活细胞密度达到最大，随后使活细胞密度降至一定程度，随后收获培养物。在一些实施方案中，生产期结束时的总活力低于约 90％、85％、80％、

75%、70%、65%、60%、55%、50%、45%、40%、35%、30%、25%、20%、15%、10%、5%。

[0164] 在一些实施方案中，在生产期期间使细胞生长确定的时间段。例如，取决于随后的生长期开始时细胞培养物的浓度、生长细胞时所处的温度和细胞的固有生长速率，可使细胞生长约 5-90 日（例如，约 5-80 日、约 5-70 日、约 5-60 日、约 5-50 日、约 5-40 日、约 5-30 日、约 5-20 日、约 5-15 日、约 5-10 日、约 10-90 日、约 10-80 日、约 10-70 日、约 10-60 日、约 10-50 日、约 10-40 日、约 10-30 日、约 10-20 日、约 15-90 日、约 15-80 日、约 15-70 日、约 15-60 日、约 15-50 日、约 15-40 日、约 15-30 日）。在一些实施方案中，生产期持续约 5、10、15、20、25、30、35、40、45、50、55、60、65、70、75、80、85 或 90 日。

[0165] 在一些实施方案中，将细胞维持在生产期，直至针对重组 I2S 蛋白的滴度达到最大。在其它实施方案中，可在该点之前收获培养物。例如，在一些实施方案中，将细胞维持在生产期，直至针对重组 I2S 蛋白的滴度达到期望的滴度。因此，针对重组 I2S 蛋白的期望平均收获滴度可以为至少 6mg 每升每日 (mg/L/ 日）（例如，至少 8、10、12、14、16、18、20、25、30、35、40、45、50、55、60、65、70、75、80、85、90、95、100、150、200、250、300、350、400、450 或 500mg/L/ 日或更多）。在一些实施方案中，针对重组 I2S 蛋白的期望平均收获滴度可在约 6-500mg/L/ 日（例如，约 6-400mg/L/ 日、约 6-300mg/L/ 日、约 6-200mg/L/ 日、约 6-100mg/L/ 日、约 6-90mg/L/ 日、约 6-80mg/L/ 日、约 6-70mg/L/ 日、约 6-60mg/L/ 日、约 6-50mg/L/ 日、约 6-40mg/L/ 日、约 6-30mg/L/ 日、约 10-500mg/L/ 日、约 10-400mg/L/ 日、约 10-300mg/L/ 日、约 10-200mg/L/ 日、约 10-100mg/L/ 日、约 10-90mg/L/ 日、约 10-80mg/L/ 日、约 10-70mg/L/ 日、约 10-60mg/L/ 日、约 10-50mg/L/ 日、约 10-40mg/L/ 日、约 10-30mg/L/ 日、约 20-500mg/L/ 日、约 20-400mg/L/ 日、约 20-300mg/L/ 日、约 20-200mg/L/ 日、约 20-100mg/L/ 日、约 20-90mg/L/ 日、约 20-80mg/L/ 日、约 20-70mg/L/ 日、约 20-60mg/L/ 日、约 20-50mg/L/ 日、约 20-40mg/L/ 日、约 20-30mg/L/ 日）的范围内。

[0166] 另外或可选择地，在一定条件下使细胞维持在生产期，所述条件使得产生的重组 I2S 蛋白达到期望的 C_{α}- 甲酰甘氨酸 (FGly) 转化百分比。在一些实施方案中，产生的重组 I2S 蛋白包含至少约 70%（例如，至少约 75%、80%、85%、90%、95%、96%、97%、98%、99%、100%）的对应于人 I2S 蛋白的 Cys59 的半胱氨酸残基至 C_{α}- 甲酰甘氨酸 (FGly) 的转化。

[0167] 另外或可选择地，在一定条件下使细胞维持在生产期，所述条件使得产生的重组 I2S 蛋白达到期望的酶活性。如本领域技术人员可理解的，重组 I2S 蛋白的酶活性可通过各种体外和体内测定来测量。在一些实施方案中，如使用肝素二糖作为底物通过体外硫酸酯释放活性测定测量的，产生的重组 I2S 蛋白的期望的酶活性为至少约 20U/mg、30U/mg、40U/mg、50U/mg、60U/mg、70U/mg、80U/mg、90U/mg 或 100U/mg。在一些实施方案中，如使用肝素二糖作为底物通过体外硫酸酯释放活性测定测量的，产生的重组 I2S 蛋白的期望的酶活性在约 20-100U/mg（例如，约 20-90U/mg、约 20-80U/mg、约 20-70U/mg、约 20-60U/mg、约 20-50U/mg、约 20-40U/mg、约 20-30U/mg、约 30-100U/mg、约 30-90U/mg、约 30-80U/mg、约 30-70U/mg、约 30-60U/mg、约 30-50U/mg、约 30-40U/mg、约 40-100U/mg、约 40-90U/mg、约 40-80U/mg、约 40-70U/mg、约 40-60U/mg、约 40-50U/mg) 的范围内。下文中提供了用于使用肝素二糖作为底物进行体外硫酸酯释放活性测定的示例性条件。通常情况下，该测定测量 I2S 从天然来源的底物肝素二糖释放硫酸根离子的能力。所释放的硫酸酯可通过离子色谱法来进

行定量。在一些情况下，离子色谱法配备有电导率检测器。作为一个非限制性实例，首先将样品缓冲交换至 10mM 乙酸钠 (pH6)，以解除配制缓冲液中磷酸根离子产生的抑制作用。随后用反应缓冲液 (10mM 乙酸钠，pH 4.4) 将样品稀释至 0.075mg/ml，并在 37℃于 30μL 反应体积中以 0.3μg I2S/100μg 底物的酶与底物比与肝素二糖温育 2 小时。然后通过在 100℃加热 3 分钟来终止反应。使用具有 IonPac AG18 保护柱的 Dionex IonPac AS18 分析柱进行分析。以 1.0 毫升 / 分钟将等度方法与 30mM 氢氧化钾一起使用，持续 15 分钟。从在 1.7 至 16.0 纳摩尔的范围内的硫酸酯标准的线性回归分析计算由 I2S 样品释放的硫酸酯的量。该报告值表达为单位每 mg 蛋白质，其中 1 个单位定义为每小时释放的 1 微摩尔的硫酸酯，并且蛋白质浓度通过 A280 测量来测定。

[0168] 在一些实施方案中，还可使用本领域已知的各种其它方法，例如测量 4- 甲基伞形酮基 - 硫酸酯至硫酸酯和天然发荧光的 4- 甲基伞形酮 (4-MUF) 的水解的 4-MUF 测定来测定重组 I2S 蛋白的酶活性。在一些实施方案中，如通过体外 4-MUF 测定测量的，产生的重组 I2S 蛋白的期望的酶活性为至少约 2U/mg、4U/mg、6U/mg、8U/mg、10U/mg、12U/mg、14U/mg、16U/mg、18U/mg 或 20U/mg。在一些实施方案中，如通过体外 4-MUF 测定测量的，产生的重组 I2S 蛋白的期望的酶活性在约 0-50U/mg(例如，约 0-40U/mg、约 0-30U/mg、约 0-20U/mg、约 0-10U/mg、约 2-50U/mg、约 2-40U/mg、约 2-30U/mg、约 2-20U/mg、约 2-10U/mg、约 4-50U/mg、约 4-40U/mg、约 4-30U/mg、约 4-20U/mg、约 4-10U/mg、约 6-50U/mg、约 6-40U/mg、约 6-30U/mg、约 6-20U/mg、约 6-10U/mg) 的范围内。下文中提供了用于进行体外 4-MUF 测定的示例性条件。通常情况下，4-MUF 测定测量 I2S 蛋白将 4- 甲基伞形酮基硫酸酯 (4-MUF-SO_4) 水解成硫酸酯和天然发荧光的 4- 甲基伞形酮 (4-MUF) 的能力。1 毫单位的活性被定义为在 37℃于 1 分钟内将 1 纳摩尔的 4-MUF-SO_4转化成 4-MUF 所需的酶的量。通常情况下，通过具有已知活性的 I2S 测试样品产生的平均荧光单位 (MFU) 可用于产生标准曲线，该曲线可被用来计算目标样品的酶活性。

[0169] 在一些实施方案中，可能有益的或必需的是在生产期期间利用营养物或已被细胞耗尽或代谢的其它培养基组分补充细胞培养物。例如，可能有利的是利用营养物或经观察在细胞培养过程中已被耗尽的其它培养基组分补充细胞培养物。可选择地或另外地，可有益的或必需的是在生产期之前补充细胞培养物。作为非限制性实例，可有益的或必需的是用氧化还原调节剂、生长调节剂 (例如，激素和 / 或其它生长因子)、特定离子 (例如钠、氯、钙、镁和磷酸根离子)、缓冲剂、维生素、核苷或核苷酸、微量元素 (通常以极低终浓度存在的无机化合物)、氨基酸、脂质或葡萄糖或其它能量来源补充细胞培养物。

[0170] 可一次将这些补充组分全部添加至细胞培养物或可以一系列添加中将它们提供给细胞培养物。在一些实施方案中，将补充组分按比例量在多个时间点提供给细胞培养物。在其它实施方案中，用这些补充组分连续供给细胞培养物。通常情况下，该过程称为灌注，并且牵涉灌注的细胞培养称为“灌注培养”。如本文中所用，术语“灌注培养”是指其中在培养过程开始后连续或半连续地给培养物提供额外的组分的培养细胞的方法。通常在连续或半连续的基础上收获培养基中的一部分细胞和 / 或组分，并且任选地对其进行纯化。

[0171] 在一些实施方案中，在生产期期间通过灌注工艺连续地交换培养基。通常情况下，每日相对于反应器的工作体积的新鲜培养基的体积 (VVD) 被定义为灌注速率。可根据本发明使用不同的灌注速率。在一些实施方案中，灌注工艺具有这样的灌注速率，以便可将添

加至细胞培养物的总体积保持在最少量。在一些实施方案中，灌注工艺具有在约 0.5-2 新鲜培养基的体积 / 反应器的工作体积 / 日 (VVD)(例如，约 0.5-1.5VVD、约 0.75-1.5VVD、约 0.75-1.25VVD、约 1.0-2.0VVD、约 1.0-1.9VVD、约 1.0-1.8VVD、约 1.0-1.7VVD、约 1.0-1.6VVD、约 1.0-1.5VVD、约 1.0-1.4VVD、约 1.0-1.3VVD、约 1.0-1.2VVD、约 1.0-1.1VVD) 的范围内的灌注速率。在一些实施方案中，灌注工艺具有约 0.5、0.55、0.6、0.65、0.7、0.75、0.8、0.85、0.9、0.95、1.0、1.05、1.10、1.15、1.2、1.25、1.3、1.35、1.4、1.45、1.5、1.55、1.6、1.65、1.7、1.75、1.8、1.85、1.9、1.95 或 2.0VVD 的灌注速率。

[0172] 灌注工艺还可通过每日每种细胞添加的新鲜培养基的体积来表征，其定义为细胞比灌注速率。可使用不同的细胞比灌注速率。在一些实施方案中，灌注工艺具有在约 0.05-5 纳升每种细胞每日 (nL/ 细胞 / 日)(例如，约 0.05-4nL/ 细胞 / 日、约 0.05-3nL/ 细胞 / 日、约 0.05-2nL/ 细胞 / 日、约 0.05-1nL/ 细胞 / 日、约 0.1-5nL/ 细胞 / 日、约 0.1-4nL/ 细胞 / 日、约 0.1-3nL/ 细胞 / 日、约 0.1-2nL/ 细胞 / 日、约 0.1-1nL/ 细胞 / 日、约 0.15-5nL/ 细胞 / 日、约 0.15-4nL/ 细胞 / 日、约 0.15-3nL/ 细胞 / 日、约 0.15-2nL/ 细胞 / 日、约 0.15-1nL/ 细胞 / 日、约 0.2-5nL/ 细胞 / 日、约 0.2-4nL/ 细胞 / 日、约 0.2-3nL/ 细胞 / 日、约 0.2-2nL/ 细胞 / 日、约 0.2-1nL/ 细胞 / 日、约 0.25-5nL/ 细胞 / 日、约 0.25-4nL/ 细胞 / 日、约 0.25-3nL/ 细胞 / 日、约 0.25-2nL/ 细胞 / 日、约 0.25-1nL/ 细胞 / 日、约 0.3-5nL/ 细胞 / 日、约 0.3-4nL/ 细胞 / 日、约 0.3-3nL/ 细胞 / 日、约 0.3-2nL/ 细胞 / 日、约 0.3-1nL/ 细胞 / 日、约 0.35-5nL/ 细胞 / 日、约 0.35-4nL/ 细胞 / 日、约 0.35-3nL/ 细胞 / 日、约 0.35-2nL/ 细胞 / 日、约 0.35-1nL/ 细胞 / 日、约 0.4-5nL/ 细胞 / 日、约 0.4-4nL/ 细胞 / 日、约 0.4-3nL/ 细胞 / 日、约 0.4-2nL/ 细胞 / 日、约 0.4-1nL/ 细胞 / 日、约 0.45-5nL/ 细胞 / 日、约 0.45-4nL/ 细胞 / 日、约 0.45-3nL/ 细胞 / 日、约 0.45-2nL/ 细胞 / 日、约 0.45-1nL/ 细胞 / 日、约 0.5-5nL/ 细胞 / 日、约 0.5-4nL/ 细胞 / 日、约 0.5-3nL/ 细胞 / 日、约 0.5-2nL/ 细胞 / 日、约 0.5-1nL/ 细胞 / 日) 的范围内的细胞比灌注速率。在一些实施方案中，灌注工艺具有约 0.05、0.1、0.15、0.2、0.25、0.3、0.35、0.4、0.45、0.5、0.55、0.6、0.65、0.7、0.75、0.8、0.85、0.9、0.95、1.0、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9、2.0、2.1、2.2、2.3、2.4、2.5、2.6、2.7、2.8、2.9、3.0、3.1、3.2、3.3、3.4、3.5、3.6、3.7、3.8、3.9、4.0、4.1、4.2、4.3、4.4、4.5、4.6、4.7、4.8、4.9 或 5.0nL/ 细胞 / 日的细胞比灌注速率。

[0173] 可在生产期期间搅拌或摇动细胞培养物，以增强充氧作用或营养物至细胞的扩散。根据本发明，本领域普通技术人员将理解，可有益的是在生长期期间控制或调节生物反应器的某些内部条件，包括但不限于 pH、温度、充氧作用等。例如，pH 可通过提供适当量的酸或碱来控制，并且充氧作用可利用本领域公知的喷雾装置来控制。还可提供一种或多种消泡剂。

[0174] 可在整个生产过程 (包括生长期、生产期和灌注) 中使用相同培养基。在一些实施方案中，在重组 I2S 的生产中使用至少 2 种不同的培养基。例如，配制用于细胞生长的营养培养基通常被用来在整个细胞生长期中支持细胞的生长，而配制用于蛋白质生产的营养培养基在生产期期间被用来支持 I2S 的表达和收获。在任一情况下，营养培养基可以包含或可以不包含血清或其它动物来源的组分 (例如，胎球蛋白)。

[0175] 根据本发明，通常使细胞悬浮生长。然而，可将细胞附着于基质。在一个实例中，可将细胞附着于悬浮于营养培养基中的微珠或粒子。

[0176] 生物反应器

[0177] 本发明还提供了用于产生重组艾杜糖 2- 硫酸酯酶的生物反应器。生物反应器可以例如是灌注、分批、补料分批、重复分批或连续型（例如，连续搅拌釜式反应器型）。通常情况下，生物反应器包括至少一个被设计和配置来装载培养基（例如，化学成分确定的营养培养基）的器皿。器皿通常还包括至少一个被设计和配置来将新鲜营养培养基流入器皿的进口。器皿通常还包括至少一个被设计和配置来将废培养基流出器皿的出口。在一些实施方案中，器皿还可包括至少一个被设计和配置来使器皿中的分离的细胞经由至少一个出口随废培养基泄放的程度最低的过滤器。还可给生物反应器配备一个或多个被设计来维持适合于细胞生长的条件的其它组件。例如，可给生物反应器配备一个或多个被设计和配置来在器皿内循环或混合营养培养基的循环或混合装置。通常情况下，将经工程化表达重组 I2S 的分离的细胞悬浮于营养培养基中。从而，在一些情况下，循环装置确保分离的细胞悬浮保留在营养培养基中。在一些情况下，将细胞附着于基质。在一些情况下，将细胞附着于一种或多种悬浮于营养培养基中的基质（例如，微珠）。生物反应器可包括一个或多个用于从器皿获得细胞悬浮液的样品的端口。可用一个或多个用于监测和 / 或控制培养条件的组件来配置生物反应器，所述培养条件包括以下条件诸如气体（例如，空气、氧气、氮气、二氧化碳）含量、流速、温度、pH 和溶解氧水平以及搅拌速度 / 循环速率。

[0178] 具有任何适当尺寸的器皿可用于生物反应器。通常情况下，器皿尺寸适合用于满足制造重组 I2S 的生产需要。在一些实施方案中，器皿被设计和配置来包含多达 1L、多达 10L、多达 100L、多达 500L、多达 1000L、多达 1500L、多达 2000L 或更多的营养培养基。在一些实施方案中，生产生物反应器的体积为至少 10L、至少 50L, 100L、至少 200L、至少 250L、至少 500L、至少 1000L、至少 1500L、至少 2000L、至少 2500L、至少 5000L、至少 8000L、至少 10, 000L 或至少 12, 000L 或更多或其间的任意体积。生产生物反应器可由对细胞生长和活力有益的，不干扰产生的 I2S 蛋白的表达或稳定性或活性的任意材料构成。示例性材料可包括但不限于玻璃、塑料或金属。

[0179] 在一些实施方案中，可将细胞培养于装载在生物器的器皿中的化学成分确定的培养基中。培养方法通常包括将新鲜营养培养基通过至少一个进口灌注入器皿和通过至少一个出口将废营养培养基从器皿泄放。泄放以高达约 0.1 个器皿体积 / 日、约 0.2 个器皿体积 / 日、约 0.3 个器皿体积 / 日、约 0.4 个器皿体积 / 日、约 0.5 个器皿体积 / 日、约 1 个器皿体积 / 日、约 1.5 个器皿体积 / 日或更高的速率进行。所述方法还包括收获包含重组 I2S 的营养培养基。收获可以以高达约 0.1 个器皿体积 / 日、约 0.2 个器皿体积 / 日、约 0.3 个器皿体积 / 日、约 0.4 个器皿体积 / 日、约 0.5 个器皿体积 / 日、约 1 个器皿体积 / 日、约 1.5 个器皿体积 / 日或更高的速率进行。灌注还通常以等于泄放速率和收获速率的总和的速率进行。例如，灌注速率可大于约 0.1、0.2、0.3、0.4、0.5、0.6、0.7、0.8、0.9、1.0、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9、2.0 个器皿体积 / 日。在一些实施方案中，灌注速率少于约 5.0、4.5、4.0、3.5、3.0、2.5、2.0、1.5、1.4、1.3、1.2、1.1、1.0、0.9、0.8、0.7、0.6、0.5 个器皿体积 / 日。示例性灌注速率描述于整个说明书中。

[0180] 监测培养条件

[0181] 在本发明的某些实施方案中，实施者可发现有益的或必需的是定期监测使细胞培养物生长的特定条件。监测细胞培养条件允许实施者确定细胞培养物是否正在以次优水平

产生重组多肽或蛋白质，或培养物是否将进入次优生产期。为了监测某些细胞培养条件，将有必要取出培养物的小等份试样进行分析。

[0182] 作为非限制实例，有益的或必需的是监测温度、pH、细胞密度、细胞活力、积分活细胞密度、克分子渗透压浓度或表达的 I2S 蛋白的滴度或活性。允许本领域普通技术人员测量这些条件的许多技术在本领域是公知的。例如，可使用血细胞计数器、库尔特计数器 (Coulter counter) 或细胞密度检查 (CEDEX) 来测量细胞密度。活细胞密度可以通过用台盼蓝 (Trypan blue) 是培养样品染色来测定。由于只有死细胞吸收台盼蓝，因此活细胞密度可通过计数细胞总数，将吸收染料的细胞数目除以细胞总数，并取倒数来确定。或者，表达的 I2S 蛋白的水平可通过标准分子生物学技术例如 SDS-PAGE 凝胶的考马斯染色、Western 印迹、Bradford 测定、Lowry 测定、Biuret 测定和 UV 吸收来测定。也可能有益地或必需的是监测表达的 I2S 蛋白的翻译后修饰，包括磷酸化和糖基化。

[0183] 表达的 I2S 蛋白的纯化

[0184] 各种方法可用于纯化或分离根据本文所述的各种方法产生的 I2S 蛋白。在一些实施方案中，表达的 I2S 蛋白被分泌至培养基中，从而可以例如通过离心或过滤（例如，作为纯化过程的第一步骤）除去细胞和其他固体。或者或另外地，将表达的 I2S 蛋白结合在宿主细胞的表面上。在本实施方案中，将表达多肽或蛋白质的宿主细胞（例如，酵母细胞）裂解以进行纯化。宿主细胞例如，酵母细胞）的裂解可以通过本领域普通技术人员公知的许多方法，包括利用玻璃珠进行的物理破碎和对高 pH 条件的暴露来实现。

[0185] 可通过标准方法（包括但不限于色谱法（例如，离子交换色谱、亲和色谱、尺寸排阻色谱和羟基磷灰石色谱）、凝胶过滤、离心或差异溶解度、乙醇沉淀）或通过任何其它可获得的用于蛋白质纯化的技术（参见，例如 Scopes, Protein Purification Principles and Practice 第 2 版，Springer-Verlag, New York, 1987 ; Higgins, S. J. 和 Hames, B.D.（编辑），Protein Expression: A Practical Approach, Oxford Univ Press, 1999 ; 和 Deutscher, M.P., Simon, M.I., Abelson, J.N.（编辑），Guide to Protein Purification: Methods in Enzymology (Methods in Enzymology Series, 第 182 卷), Academic Press, 1997，将所有参考资料通过引用并入本文）分离和纯化 I2S 蛋白。对于具体地免疫亲和色谱法，可通过将蛋白质结合于包含抗体的亲和柱来使其分离，所述抗体是针对该蛋白质产生的并且被附着至固定载体。或者，可通过标准重组技术将亲和标签例如流感病毒衣壳蛋白序列、多聚组氨酸或谷胱甘肽 -S- 转移酶连接于蛋白质，以使其通过在适当的亲和柱上通过来容易地纯化。可以在任何或所有阶段添加蛋白酶抑制剂，例如苯基甲基磺酰氟 (PMSF)、亮肽素、胃酶抑素或抑肽酶以在纯化过程中减少或消除所述多肽或蛋白质的降解。当必需裂解细胞以分离和纯化表达的多肽或蛋白质时，特别想要蛋白酶抑制剂。

[0186] 示例性纯化方法描述于下面的实施例部分中。另外的纯化方法描述于与其同一日提交的标题为“重组 I2S 蛋白的纯化 (Purification of Recombinant I2S Protein)”的临时申请中，所述临时申请的全部公开内容通过引用并入本文。

[0187] 药物组合物和施用

[0188] 可按照已知的方法向亨特综合症患者施用纯化的重组 I2S 蛋白。例如，可静脉内、皮下、肌内、肠胃外、透皮或透粘膜（例如，经口或经鼻））递送纯化的重组 I2S 蛋白。

[0189] 在一些实施方案中，可通过静脉内施用向受试者施用重组 I2S 或包含其的药物组合物。

[0190] 在一些实施方案中，通过鞘内施用向受试者施用重组 I2S 或包含其的药物组合物。如本文中所用的，术语“鞘内施用”或“鞘内注射”是指至椎管（围绕脊髓的鞘内空间）内的注射。可使用各种技术，包括但不限于，通过钻孔的侧脑室注射或池穿刺或腰椎穿刺等。在一些实施方案中，根据本发明的“鞘内施用”或“鞘内递送”是指通过腰部区或区域的 IT 施用或递送，即腰 IT 施用或递送。如本文中所用，术语“腰部区域”或“腰部区”指的是第三和第四腰（下背部）椎骨之间的区，更包含地，脊椎的 L2-S1 区域。

[0191] 在一些实施方案中，通过皮下（即皮肤下方）向受试者施用重组 I2S 或包含其的药物组合物。为此目的，可使用注射器注射制剂。然而，用于该制剂的施用的其它装置是可获得的，例如注射装置（例如，Inject-ease™和 Genject ™装置）；注射器笔（例如 GenPen™）；无针装置（例如，MediJector™和 BioJector ™）；和皮下贴片递送系统。

[0192] 在一些实施方案中，可将鞘内施用与其他施用途径（例如，静脉内、皮下、肌内、肠胃外、透皮或透粘膜（例如，经口或经鼻））一起使用。

[0193] 本发明设想了治疗有效量的本文中描述的重组 I2S 或包含所述重组 I2S 的药物组合物的单次以及多次施用。可定期施用重组 I2S 或包含其的药物组合物，这取决于受试者的病况（例如，溶酶体贮积病）的性质、严重性和程度。在一些实施方案中，可定期（例如，每年一次，每六个月一次，每五个月一次，每三个月一次，每两月一次（每两个月一次），每月一次（每个月一次），每两周一次（每隔两周一次），每周一次，每日一次或连续地）周期性施用治疗有效的量的重组 I2S 或包含其的药物组合物。

[0194] 可用生理上可接受的载体或赋形剂配制重组 I2S 或包含其的药物组合物以制备药物组合物。载体和治疗剂可以是无菌的。所述制剂应该适合施用模式。

[0195] 适合的药学上可接受的载体包括但不限于：水、盐溶液（例如，NaCl）、盐水、缓冲盐水、醇、甘油、乙醇、阿拉伯胶、植物油、苄醇、聚乙二醇、明胶、碳水化合物如乳糖、直链淀粉或淀粉、糖例如甘露醇、蔗糖或其他糖、葡萄糖、硬脂酸镁、滑石、硅酸、粘性石蜡、芳香油、脂肪酸酯、羟甲基纤维素、聚乙烯吡咯烷酮等及其组合。必要时，可将药物制剂与助剂（例如，润滑剂、防腐剂、稳定剂、润湿剂、乳化剂、用于影响渗透压的盐、缓冲剂、着色剂、调味剂和 / 或芳香物质等）混合，所述助剂其不与活性化合物产生不利反应或干扰它们的活性。在一些实施方案中，使用适合于静脉内施用的水溶性载体。

[0196] 必要时，组合物或药剂还可包含少量润湿剂或乳化剂或 pH 缓冲剂。组合物可以是液体溶液、悬浮液、乳液、片剂、丸剂、胶囊剂、持续释放制剂或粉剂。还可利用常规粘合剂和载体如甘油三酯将组合物配制成栓剂。口服制剂可以包含标准载体如药物级的甘露醇、乳糖、淀粉、硬脂酸镁、聚乙烯吡咯烷酮、糖精钠、纤维素、碳酸镁等。

[0197] 可按照常规程序将组合物或药剂配制为适于向人类施用的药物组合物。例如，在一些实施方案中，用于静脉内施用的组合物通常是在无菌等渗含水缓冲液中的溶液。必要时，组合物还可以包含增溶剂和局部麻醉剂以减轻注射部位的疼痛。通常，单独地提供成分或以单位剂型将成分混合在一起，例如，作为标明活性剂的量的密闭容器例如安瓿或小袋中的干燥的冻干粉剂或无水浓缩物。当将通过输注施用组合物时，可用含有无菌药用级水、盐水或右旋糖 / 水的输液瓶分配所述组合物。当通过注射施用组合物时，可提供一安瓿的

注射用无菌水或盐水以便可在施用前混合成分。

[0198] 如本文中所用，主要基于本发明的药物组合物中包含的治疗剂的总量确定术语“治疗有效量”。通常，治疗有效量足以对受试者实现有意义的益处（例如，治疗、调节、治愈、预防和/或改善潜在的疾病或病症）。例如，治疗有效量可以是足以实现期望的治疗和/或预防效果的量，例如足以调节溶酶体酶受体或它们的活性，从而治疗这样的溶酶体贮积病或其症状（例如，在向受试者施用本发明的组合物后减少或消除“斑状体”或细胞空泡形成的存在或发生率）的量。通常地，向需要其的受试者施用的治疗剂（例如，重组溶酶体酶）的量将取决于受试者的特征。这样的特征包括受试者的状态、疾病的严重度、一般健康状况、年龄、性别和体重。取决于这些和其他相关因素，本领域普通技术人员将能够容易地确定适当的剂量。此外，可任选地将客观和主观测定用于鉴定最佳剂量范围。

[0199] 通常在可包括多个单位剂量的给药方案中施用治疗有效量。对于任何特定的治疗性蛋白，治疗有效量（和/或有效给药方案内的适当单位剂量）可以例如取决于施用途径、取决于与其它药剂的组合而变化。同样地，对于任何特定患者的具体的治疗有效量（和/或单位剂量）还可取决于多种因素，包括被治疗的病症和病症的严重程度；所使用的具体药剂的活性；使用的具体组合物；患者的年龄、体重、一般健康状况、性别和饮食；施用时间、施用途径和/或使用的特定融合蛋白的排泄或代谢速率；治疗的持续时间；以及在医学领域中公知的类似因素。

[0200] 另外的示例性药物组合物和施用方法描述于标题为“用于艾杜糖-2-硫酸酯酶的CNS递送的方法和组合物(Methods and Compositions for CNS Delivery of Iduronate-2-Sulfatase)”的PCT公布WO2011/163649；和2012年3月30日提交的标题为“艾杜糖醛酸2硫酸酯酶的皮下施用(Subcutaneous administration of iduronate 2sulfatase)”的临时申请序列号61/618,638，将这两者的全部公开内容通过引用并入本文。

[0201] 应当进一步理解，对于任何特定的受试者，应当根据个体需要和施用或监督酶替代疗法的施用的人的专业判断来随时间调整具体剂量方案，并且本文中所陈述的剂量范围仅是示例性的并且无意限定本发明的范围或实践。

实施例

[0202] 实施例1. 共表达重组I2S和FGE的最优化的细胞系的产生

[0203] 本实施例举例说明可用于产生重组I2S蛋白的共表达重组I2S和FGE的示例性细胞系。对于本领域技术人员将很明确的是许多替代方法、表达载体和克隆技术是可获得的。

[0204] 人艾杜糖的2-硫酸酯酶(I2S)的典型成熟形式是525个氨基酸的糖蛋白，其经历大量的加工和翻译后修饰以进行酶的激活，如糖基化和半胱氨酸至甲酰甘氨酸的转化（图1）。在哺乳动物细胞中，I2S酶内的保守半胱氨酸残基（即，在氨基酸59上）通过甲酰甘氨酸生成酶(FGE)被转化为甲酰甘氨酸。I2S酶的活性位点内的半胱氨酸至甲酰甘氨酸的转化是产生人硫酸酯酶的活性形式中的一个重要步骤。本实验的目的是工程化共表达I2S和FEG的最优化人细胞系以用于产生活性重组I2S。

[0205] 图2举例说明了许多用于共表达I2S和FGE的示例性构建体设计。例如，I2S和FGE的表达单位可以位于单独的载体，并且可共转染或单独转染单独的载体（图2A）。或

者，I2S 和 FGE 的表达单位可以位于同一载体（图 2B）。在一种配置中，I2S 和 FGE 可以在相同的载体上，但在单独的启动子控制下，也被称为单独的顺反子（图 2B(1)）。或者，I2S 和 FGE 可以设计为转录连接的顺反子，即，I2S 和 FGE 被设计为在相同启动子控制下的一个开放阅读框（图 2B(2)）。通常情况下，内部核糖体进入位点（IRES）被设计来允许在信使 RNA(mRNA) 的中部起始翻译（图 2B(2)）。

[0206] 将人细胞系工程化以共表达具有 SEQ ID NO :2 中显示的氨基酸序列的人 I2S 蛋白和具有 SEQ ID NO :6 中显示的氨基酸序列的人甲酰甘氨酸生成酶 (FGE)。

[0207] SEQ ID NO :2

[0208] ＞全长前体艾杜糖 -2- 硫酸酯酶

[0209] MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLAS HSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHT DDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAV GYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVA|YNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQS YFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEK LFPYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYL PGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPL QDHNMYNDSQGGDLFQLLMP

[0210] SEQ ID NO :6

[0211] 全长人 FGE 前体 :

[0212] MAAPALGLVCGRCPELGLVLLLLLLSLLCGAAGSQEAGTGAGAGSLAGSCGCGTPQRPGAHGSSAAAHR YSREANAPGPVPGERQLAHSKMVPIPAGVFTMGTDDPQIKQDGEAPARRVTIDAFYMDAYEVSNTEFEKFVNSTGYL TEAEKFGDSFVFEGMLSEQVKTNIQQAVAAAPWWLPVKGANWRHPEGPDSTILHRPDHPVLHVSWNDAVAYCTWAGK RLPTEAEWEYSCRGGLHNRLFPWGNKLQPKGQHYANIWQGEFPVTNTGEDGFQGTAPVDAFPPNGYGLYNIVGNAWE WTSDWWTVHHSVEETLNPKGPPSGKDRVKKGGSYMCHRSYCYRYRCAARSQNTPDSSASNLGFRCAADRLPTMD

[0213] 通过人 CMV 启动子控制 I2S 和 FGE 表达。I2S mRNA 的翻译导致 550 个氨基酸的全长 I2S 蛋白 (SEQ ID NO:2) 的合成，所述蛋白质包含 25 个氨基酸的信号肽。除去信号肽，并且从细胞分泌可溶性酶。

[0214] 将细菌新霉素磷酸转移酶 (neo) 编码序列和 / 或杀稻瘟菌素 S(Blasticidin S) 脱氨酶 (BSD) 基因用于使得能够分别使用新霉素类似物 G418 和 / 或杀稻瘟菌素选择转染的细胞。此外，在 I2S 和 / 或 FGE 编码载体上使用小鼠二氢叶酸还原酶 (DHFR) 基因以允许通过甲氨蝶呤 (MTX) 选择来分离包含增加的拷贝的 I2S 和 / 或 FGE 编码序列的细胞系。

[0215] 分离产生 I2S 的细胞，并使其经历适当的药物选择来分离具有增加的拷贝数的转染的 I2S 和 / 或 FGE 基因的细胞。通过 ELISA 进行 I2S 的定量。

[0216] 也使细胞群在甲氨蝶呤 (MTX) 中经历分步选择以分离具有增加的 I2S 生产率的细胞。在 MTX 选择期间通过 ELISA 监控 I2S 的生产率。

[0217] 经过多轮扩增后，随后通过从包含 10％小牛血清的 DMEM 逐步减少至无血清的化学成分确定的培养基，使几个产生 I2S 的克隆在无血清培养基中经历悬浮培养适应。通过有限稀释克隆建立几个单独的克隆群体。通过 I2S 酶活性测定和 ELISA 筛选菌落。两个稳定的细胞系 2D 和 4D 显示高百分比的活力和 I2S 的强劲表达，并且被选择用于进一步开发。

[0218] 实施例 2. 无血清悬浮细胞培养

[0219] 本实施例证明可将无血清细胞培养系统用于成功地培养共表达 I2S 和 FGE 的细胞系以产生重组 I2S。

[0220] 产生种子培养物

[0221] 简言之，使用实施例 1 的 2D 或 4D 细胞系建立种子培养物。将细胞转移至 250ml 装有无血清化学成分确定的扩增培养基（补充有甲氨蝶呤（以用于选择），用碳酸氢钠调整至 pH 7.3）的通风组织培养摇瓶，并在标准条件下生长。

[0222] 细胞培养扩增

[0223] 在达到期望的活细胞密度后，将初始种子培养物用于接种由 500ml 组织培养摇瓶，随后 2x 1L 组织培养摇瓶组成的一系列分步细胞培养扩增的第一组织培养摇瓶。在每一种情况下，在达到期望的细胞密度后，将先前的细胞培养物以其整体转移来接种随后更大的培养瓶。

[0224] 通过将每一个 2x1L 的培养物转移到 10L Cellbag bioreactor® (Wave Europe)，并添加扩增培养基至 2.5kg 的终重量来进行分批培养扩增。在达到期望的细胞密度后，添加新的扩增培养基至 5.0kg 的终重量，并使细胞生长至期望的密度。将 10L Cellbag 转移到 Wav e bioreactor® 系统 (Wave Europe)，并改变培养条件以允许在连续培养基灌注的条件下生长。以 5.0L/ 日 (1.0vvd) 的靶重量递送扩增生长培养基，并收集样品以进行 pH、谷氨酰胺、谷氨酸、葡萄糖、铵、乳酸盐、pCO_2和克分子渗透压浓度的离线代谢物分析。

[0225] 在达到期望的细胞密度后，将整个 10L 的细胞培养物转移至包含 20kg 新鲜的扩增培养基的 50L Wave Cellbag bioreactor®，并再次生长至期望的细胞密度。

[0226] 生物反应器扩增

[0227] 随后使用 200L 的一次性生物反应器和离心灌注装置（Centritech® CELL II 单位，Pneumatic Scale Corporation）进行细胞扩增，所述装置被设计来浓缩细胞和澄清培养基，以在灌注介导的细胞培养过程中再循环。用足以获得期望的细胞密度的 50L 培养物的一部分接种扩增培养基。

[0228] 随后，将 200L 培养物的一部分用于接种 2000L 一次性生物反应器和离心机灌注装置（Centritech® CELL II 单位，Pneumatic Scale Corporation）中。使细胞在分批生长条件下生长 2 天。在为期 2 天的生长后，调整条件以进行连续灌注，从而开始过渡期的起始。

[0229] 生物反应器生产

[0230] 对于生产期，使用 2 个 Centritech CELL II 单位。在过渡期开始后约 24 小时（细胞通常在该时间点已达到期望的细胞密度）开始生产期。通过调节泄放速率来维持细胞密度持续期望的生产时间。

[0231] 实施例 3. 无血清细胞培养中产生的重组 I2S 酶的生理化学和生物学表征

[0232] 本实施例的目的是进行使用上述无血清细胞培养法产生的重组 I2S 蛋白的详尽表征。

[0233] SDS-PAGE

[0234] 关于本实验，使用上述方法在两个单独的无血清细胞培养反应中，使用 2D 和 4D 人细胞系产生重组 I2S 蛋白。在生产期期间收集样品，通过 SDS-PAGE 分析纯化的 I2S 酶，并

用银染处理以使其可视。图 3 显示，在每一个单独的制造实验中，在无血清条件下从 2D 和 4D 细胞系产生的 I2S 蛋白迁移适当的尺寸（泳道 5 和 6）上，如在与蛋白质分子量标准（泳道 1）和商购可得 I2S 测定对照（泳道 2 和 3）比较后指示的。此外，在无血清条件（泳道 5 和 6）下产生的重组 I2S 也迁移与 I2S 参考标准（泳道 4）相同的尺寸。

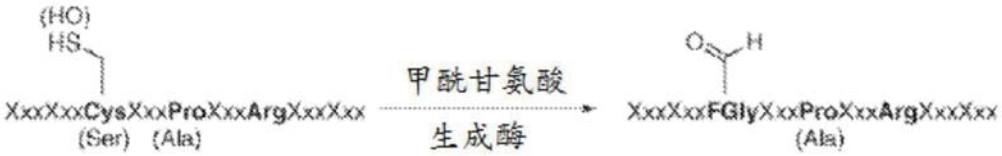
[0235] 肽图谱

[0236] 使用在上述无血清培养条件下生长的 I2S-AF 2D 细胞系产生重组 I2S 蛋白。将从 I2S-AF 2D 细胞系产生的分离的重组 I2S 和参考人 I2S 的样品各自经历蛋白质水解消化（例如，通过胰蛋白酶），并且通过 HPLC 分析进行检查。示例性结果示于图 4 中。

[0237] 甲酰甘氨酸转化百分比

[0238] 肽图谱可用于确定 FGly 转化百分比。I2S 活化需要通过甲酰甘氨酸生成酶 (FGE) 进行的半胱氨酸（对应于成熟人 I2S 的位置 59）至甲酰甘氨酸的转化，如下文中显示的：

[0239]



[0240] 因此，甲酰甘氨酸转化的百分比（% FG）可以使用下列公式计算：

[0241]

$$\%FG\ (DS的) = \frac{活性I2S分子的数目}{总的(活性+无活性)I2S分子的数目} \times 100$$

[0242] 例如，50％ FG 意指一半纯化的重组 I2S 无酶活性而没有任何治疗效果。

[0243] 肽图谱用于计算％ FG。简言之，使用蛋白酶（例如，胰蛋白酶或糜蛋白酶）将重组 I2S 蛋白消化成短肽。使用 HPLC 分离和表征短肽。表征包含对应于成熟人 I2S 的位置 59 的位置的肽以确定相较于对照（例如，无 FGly 转化的 I2S 蛋白或具有 100％ FGly 转化的 I2S 蛋白）位置 59 上的 Cys 是否被转化成 FGly。可基于对应的峰面积测定包含 FGly 的肽的量（对应于活性 I2S 分子的数目）和具有 FGly 和 Cys 的肽的总量（对应于总的 I2S 分子的数目），并计算反映％ FG 的比率。示例性结构示于表 4 中。

[0244] 聚糖图谱 - 甘露糖 -6 磷酸和唾液酸含量

[0245] 测定在无血清细胞培养条件下产生的重组 I2S 蛋白的聚糖和唾液酸组成。使用阴离子交换色谱法进行聚糖组成的定量。如下所述，在这些条件下产生的重组 I2S 的聚糖图谱由七个峰群组成，根据递增量的负电荷（至少部分地因酶消化而从唾液酸和甘露糖 -6- 磷酸糖型产生）洗脱。简言之，使用如下方式处理利用无血清细胞培养方法 (I2S-AF2D 无血清和 I2S-AF 4D 无血清）获得的纯化的重组 I2S 和产生的参照重组 I2S：(1) 使用纯化的神经氨酸酶（分离自产脲节杆菌 (Arthrobacter Ureafaciens)(10mU/μL)，Roche Biochemical(Indianapolis,IN)，目录号 269611(1U/100μL)）以除去唾液酸残留物，(2) 在 37±1℃用碱性磷酸酶处理 2 小时以完全释放甘露糖 -6- 磷酸残留物，(3) 碱性磷酸酶 + 神经氨酸酶或 (4) 无处理。使用配备有 Dionex CarboPac PA1 保护柱的 CarboPac PA1 分析柱，通过利用脉冲安培检测 (HPAE-PAD) 的高效阴离子交换色谱法分析每一种酶消

化。对于每一种测定，运行一系列在 0.4 至 2.0 纳摩尔的范围内的唾液酸和甘露糖 -6- 磷酸标准。在环境柱温度下将使用在 100mM 氢氧化钠中的 48mM 乙酸钠的等度方法以 1.0 毫升 / 分钟的流速运行至少 15 分钟，以洗脱每个峰。将从每个单独的运行产生的 I2S-AF 和参照 I2S 样品的数据分别组合成单个色谱来表示每个相应的重组蛋白的聚糖图谱。如图 5 中显示的，使用人细胞系无血清法产生的 I2S 的示例性聚糖图谱显示构成中性、单、双唾液酸化、单磷酸化、三唾液酸化和混合（单唾液酸化和封端的甘露糖 -6- 磷酸）、四唾液酸化和混合（双唾液酸化和封端的甘露糖 -6- 磷酸）以及二磷酸化聚糖的代表性洗脱峰（按洗脱顺序）。

[0246] 从唾液酸标准的线性回归分析计算每一个重组 I2S 样品的平均唾液酸含量（摩尔唾液 / 摩尔蛋白质）。使用 PeakNet 6 软件使每一个色谱图运行可见。唾液酸标准和从重组 I2S 测定对照和测试样品释放的唾液酸显现为单个峰。使用下列公式将 I2S 的唾液酸（纳摩尔）的量计算为原始值：

[0247]

$$\text{唾液酸(摩尔/摩尔}I2S) = \frac{(\text{纳摩尔唾液酸})}{(0.3272)(C)}$$

[0248] 其中 C 为样品或重组 I2S 测定对照的蛋白质浓度（以 mg/ml 表示）。

[0249] 使用下列公式计算每一个测试样品的表示为每摩尔蛋白质的唾液酸的摩尔数的唾液酸的校正值：

[0250]

$$\text{校正的唾液酸} = \frac{(\text{样品原始唾液酸值})x(\text{确定的艾杜糖硫酸酯酶测定对照值})}{(\text{艾杜糖硫酸酯酶测定原始唾液酸值})}$$

[0251] 表示由 I2S-AF 2D 或 4D 细胞系产生的重组 I2S 上的唾液酸含量的示例性数据示于表 4 中。

[0252] 表 4: 无血清细胞培养物中产生的重组 I2S 的示例性表征

[0253]

测定	I2S-AF 2D (无血清)
肽图谱	
L1	101
L10	100
L12	102
L13	97
L14	101
L17	100
L20	102
宿主细胞蛋白质	< 62.5 ng/mg
离子交换 HPLC%面积	
峰 A	62
峰 A+B	82
Peak E+F	0
甲酰甘氨酸%	87
比活性(U/mg)(硫酸酯释放测定)	64
尺寸排阻 HPLC%	≥99.8
聚糖图谱	
单唾液酸化的	105
二唾液酸化的	93
单磷酸化的	139
三唾液酸化的	89
四唾液酸化的	125

[0254]

二磷酸化的	95
唾液酸(mol/mol)	20

[0255] 比活性

[0256] 使用体外硫酸酯释放测定或 4-MUF 测定分析在无血清细胞培养条件下使用 2D 和 4D 细胞系产生的重组 I2S 酶的比活性。

[0257] 体外硫酸酯释放活性测定

[0258] 体外硫酸酯释放活性测定使用肝素二糖作为底物来进行。具体地，该测定测量 I2S 从天然来源的底物肝素二糖释放硫酸根离子的能力。释放的硫酸酯可通过配备有电导率检测器的离子色谱来定量。简言之，首先将样品缓冲交换至 10mM 醋酸钠，pH 为 6 以消除由配制缓冲液中的磷酸根离子产生的抑制。随后用反应缓冲液 (10mM 醋酸钠，pH 值 4.4) 将样品稀释至 0.075mg/ml，并在 37℃于 30 μL 反应体积中以 0.3 μg I2S/100 μg 底物的酶与底物的比率与肝素二糖一起温育 2 小时。随后通过在 100℃加热样品 3 分钟来终止反应。使用

具有 IonPac AG18 保护柱的 Dionex IonPac AS18 分析柱进行分析。以 1.0 毫升 / 分钟利用 30mM 氢氧化钾来使用等度方法，进行 15 分钟。根据在 1.7 至 16.0 纳摩尔的范围内的硫酸酯标准的线性回归分析计算通过 I2S 样品释放的硫酸酯的量。可报告值表示为每 mg 蛋白质的单位，其中 1 个单位被定义为 1 微尔的每小时释放的硫酸酯，并且通过 A280 测量来测定蛋白质浓度。示例性结果示于表 4 中。

[0259] 4-MUF 测定

[0260] 还可使用基于荧光的 4-MUF 测定来分析在无血清细胞培养条件下使用 2D 和 4D 细胞系产生的重组 I2S 酶的比活性。简言之，该测定测量了 I2S 底物 4- 甲基伞形酮基硫酸酯 (4-MUF-SO_4) 的水解。在 4-MUF-SO_4底物被 I2S 切割后，该分子被转化成硫酸酯和天然发荧光的 4- 甲基伞形酮 (4-MUF)。作为结果，I2S 酶活性可以通过评价随时间的荧光信号的整体变化来测定。对于本实验，使从 I2S-AF 2D 和 4D 人细胞系产生的纯化的 I2S 酶与 4- 甲基伞形酮基硫酸酯 (4-MUF-SO_4钾盐 (Sigma 目录号 M-7133) 温育。使用以 1:100、1:200 和 1:20,000 的原液稀释的商购可得的 I2S 酶，利用一系列对照参照样品进行测定的校准。在 37℃运行酶测定，并使用校准的荧光计进行测定。使用对于每个参考标准获得的荧光值，使用下列公式确定变异系数百分比 ：

[0261]

$$\%CV = \frac{\text{原始荧光值的标准差}(N=3)}{\text{平均荧光值}} X\,100\%$$

[0262] 随后使用下列公式将百分比 CV 值用于计算每一个样品的修正平均荧光，以确定以 mU/mL 表示的报告酶的活性 ：

[0263]

$$mU/mL = (CFU)\left(\frac{1\text{纳摩尔}/L}{10\,FU}\right)\left(\frac{1L}{10^3 mL}\right)\left(\frac{2.11mL}{0.01mL}\right)\left(\frac{1\text{小时}}{60\text{分钟}}\right)\left(\frac{1mU}{\text{纳摩尔}}\right)(DF)$$

[0264] CFU ＝负修正平均荧光

[0265] DF - 稀释因子

[0266] 1 毫单位的活性是在 37℃于 1 分钟内将 1 纳摩尔的 4- 甲基伞形酮基硫酸酯转化为 4- 甲基伞形酮所需的酶的量。

[0267] 电荷特征谱

[0268] 利用高效液相色谱 (HPLC) 系统，通过强阴离子交换 (SAX) 色谱测定每一种纯化的重组 I2S 的电荷分布。该方法基于表面电荷差异分离样品内的重组 I2S 变体。在 pH 8.00 时，带负电荷的物质吸附至 SAX 柱的固定正电荷上。将递增离子强度的梯度用于以与它们与柱的离子相互作用的强度成比例的方式洗脱每一个蛋白质物质。将 100 微克的纯化的 I2S(从在无血清生长条件下的 2D 细胞系分离的) 或参考重组 I2S 酶上样至在环境温度下保持的并且被平衡至 20mM Tris-HC1，pH8.00 的 Amersham Biosciences Mini Q PE(4.6x 50mm) 柱上。使用 20mM Tris-HC1，1.0M 氯化钠，pH 8.00 的流动相，以 0.80 毫升 / 分钟的流速进行梯度洗脱。通过测量样品洗脱液在 280nm 波长处的吸光度来在运行过程中连续测定蛋白质浓度。示例性结果示于图 6 中。

[0269] 虽然本文描述的某些化合物、组合物和方法已经根据某些实施方案进行特定的描

述，但是下列实施例仅用于说明本发明的化合物，并且无意对其进行限制。

[0270] 如本文在说明书中和权利要求书中使用的冠词“一个 (a)”和“一种 (an)”，除非清楚地表明与之相反，否则应该被理解为包括其复数个指示物。在某组的一个或多个成员之间包括“或者”的权利要求或者描述，被认为：如果一个、多于一个或所有组成员存在、被采用或者以其它方式与给定的产品或过程相关，那么就是符合的；除非指示相反或者以其它方式从上下文中明显可见。本发明包括这样的实施方案：其中所述组的只有一个成员存在、被采用或者以其它方式与给定的产品或过程相关。本发明也包括这样的实施方案：其中多于一个或者整个组成员存在、被采用或者以其他方式与给定的产品或过程相关。而且，应该理解，本发明涵盖来自一条或多条所列出的权利要求所有的变型、组合以及排列，其中一种或多种限制、元素、条款、描述性术语等根据所述相同基础权利要求（或者相关、任意其它权利要求）被引入到另一权利要求，除非以其它方式指出或者除非其对于本领域技术人员将明显地出现矛盾或者不一致。当元素以列表形式呈现（例如，在 Markush 组中或者类似的形式）时，应该理解，所述元素的每个亚组也被公开，并且任何元素可以从所述组删除。应该理解，一般而言，当本发明、或者本发明的某些方面，被指出包括特定的元素、特征等时，那么本发明或者本发明的方面的某些实施方案由这些元素、特征等组成或基本由其组成。为了鉴定起见，本文没有在每种情形中用过多的词汇明确地陈述那些实施方案。还应该理解，本发明的任何实施方案或者方面可以被明确地从所述权利要求排除，无论所述具体的排除是否在本说明书中叙述。本文所引用的出版物、网址和其它参考材料，为了描述本发明的背景以及提供关于其实践的其它细节，都通过引用并入本文。

[0001]

序列表

<110> C•张
F•博尔戈

<120> 产生重组艾杜糖2-硫酸酯酶的方法

<130> 2006685-0339

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<400> 1

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Phe Gln Asn Ala Phe Ala Gln Gln Ala Val Cys Ala Pro Ser Arg Val
50 55 60

Ser Phe Leu Thr Gly Arg Arg Pro Asp Thr Thr Arg Leu Tyr Asp Phe
65 70 75 80

Asn Ser Tyr Trp Arg Val His Ala Gly Asn Phe Ser Thr Ile Pro Gln
85 90 95

Tyr Phe Lys Glu Asn Gly Tyr Val Thr Met Ser Val Gly Lys Val Phe
100 105 110

His Pro Gly Ile Ser Ser Asn His Thr Asp Asp Ser Pro Tyr Ser Trp
115 120 125

Ser Phe Pro Pro Tyr His Pro Ser Ser Glu Lys Tyr Glu Asn Thr Lys

[0002]

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Thr Cys Arg Gly Pro Asp Gly Glu Leu His Ala Asn Leu Leu Cys Pro
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Val Asp Val Leu Asp Val Pro Glu Gly Thr Leu Pro Asp Lys Gln Ser
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Thr Glu Gln Ala Ile Gln Leu Leu Glu Lys Met Lys Thr Ser Ala Ser
180 185 190

Pro Phe Phe Leu Ala Val Gly Tyr His Lys Pro His Ile Pro Phe Arg
195 200 205

Tyr Pro Lys Glu Phe Gln Lys Leu Tyr Pro Leu Glu Asn Ile Thr Leu
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Ala Pro Asp Pro Glu Val Pro Asp Gly Leu Pro Pro Val Ala Tyr Asn
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Ser Val Pro Tyr Gly Pro Ile Pro Val Asp Phe Gln Arg Lys Ile Arg
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Gln Ser Tyr Phe Ala Ser Val Ser Tyr Leu Asp Thr Gln Val Gly Arg
275 280 285

Leu Leu Ser Ala Leu Asp Asp Leu Gln Leu Ala Asn Ser Thr Ile Ile
290 295 300

Ala Phe Thr Ser Asp His Gly Trp Ala Leu Gly Glu His Gly Glu Trp
305 310 315 320

Ala Lys Tyr Ser Asn Phe Asp Val Ala Thr His Val Pro Leu Ile Phe
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Tyr Val Pro Gly Arg Thr Ala Ser Leu Pro Glu Ala Gly Glu Lys Leu
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Phe Pro Tyr Leu Asp Pro Phe Asp Ser Ala Ser Gln Leu Met Glu Pro

[0003]

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Gly Arg Gln Ser Met Asp Leu Val Glu Leu Val Ser Leu Phe Pro Thr
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Leu Ala Gly Leu Ala Gly Leu Gln Val Pro Pro Arg Cys Pro Val Pro
385 390 395 400

Ser Phe His Val Glu Leu Cys Arg Glu Gly Lys Asn Leu Leu Lys His
405 410 415

Phe Arg Phe Arg Asp Leu Glu Glu Asp Pro Tyr Leu Pro Gly Asn Pro
420 425 430

Arg Glu Leu Ile Ala Tyr Ser Gln Tyr Pro Arg Pro Ser Asp Ile Pro
435 440 445

Gln Trp Asn Ser Asp Lys Pro Ser Leu Lys Asp Ile Lys Ile Met Gly
450 455 460

Tyr Ser Ile Arg Thr Ile Asp Tyr Arg Tyr Thr Val Trp Val Gly Phe
465 470 475 480

Asn Pro Asp Glu Phe Leu Ala Asn Phe Ser Asp Ile His Ala Gly Glu
485 490 495

Leu Tyr Phe Val Asp Ser Asp Pro Leu Gln Asp His Asn Met Tyr Asn
500 505 510

Asp Ser Gln Gly Gly Asp Leu Phe Gln Leu Leu Met Pro
515 520 525

<210> 2
<211> 550
<212> PRT
<213> 智人

<400> 2

Met Pro Pro Pro Arg Thr Gly Arg Gly Leu Leu Trp Leu Gly Leu Val
1 5 10 15

Leu Ser Ser Val Cys Val Ala Leu Gly Ser Glu Thr Gln Ala Asn Ser
20 25 30

[0004]

Thr Thr Asp Ala Leu Asn Val Leu Leu Ile Ile Val Asp Asp Leu Arg
35 40 45

Pro Ser Leu Gly Cys Tyr Gly Asp Lys Leu Val Arg Ser Pro Asn Ile
50 55 60

Asp Gln Leu Ala Ser His Ser Leu Leu Phe Gln Asn Ala Phe Ala Gln
65 70 75 80

Gln Ala Val Cys Ala Pro Ser Arg Val Ser Phe Leu Thr Gly Arg Arg
85 90 95

Pro Asp Thr Thr Arg Leu Tyr Asp Phe Asn Ser Tyr Trp Arg Val His
100 105 110

Ala Gly Asn Phe Ser Thr Ile Pro Gln Tyr Phe Lys Glu Asn Gly Tyr
115 120 125

Val Thr Met Ser Val Gly Lys Val Phe His Pro Gly Ile Ser Ser Asn
130 135 140

His Thr Asp Asp Ser Pro Tyr Ser Trp Ser Phe Pro Pro Tyr His Pro
145 150 155 160

Ser Ser Glu Lys Tyr Glu Asn Thr Lys Thr Cys Arg Gly Pro Asp Gly
165 170 175

Glu Leu His Ala Asn Leu Leu Cys Pro Val Asp Val Leu Asp Val Pro
180 185 190

Glu Gly Thr Leu Pro Asp Lys Gln Ser Thr Glu Gln Ala Ile Gln Leu
195 200 205

Leu Glu Lys Met Lys Thr Ser Ala Ser Pro Phe Phe Leu Ala Val Gly
210 215 220

Tyr His Lys Pro His Ile Pro Phe Arg Tyr Pro Lys Glu Phe Gln Lys
225 230 235 240

Leu Tyr Pro Leu Glu Asn Ile Thr Leu Ala Pro Asp Pro Glu Val Pro
245 250 255

[0005]

Asp Gly Leu Pro Pro Val Ala Tyr Asn Pro Trp Met Asp Ile Arg Gln
260 265 270

Arg Glu Asp Val Gln Ala Leu Asn Ile Ser Val Pro Tyr Gly Pro Ile
275 280 285

Pro Val Asp Phe Gln Arg Lys Ile Arg Gln Ser Tyr Phe Ala Ser Val
290 295 300

Ser Tyr Leu Asp Thr Gln Val Gly Arg Leu Leu Ser Ala Leu Asp Asp
305 310 315 320

Leu Gln Leu Ala Asn Ser Thr Ile Ile Ala Phe Thr Ser Asp His Gly
325 330 335

Trp Ala Leu Gly Glu His Gly Glu Trp Ala Lys Tyr Ser Asn Phe Asp
340 345 350

Val Ala Thr His Val Pro Leu Ile Phe Tyr Val Pro Gly Arg Thr Ala
355 360 365

Ser Leu Pro Glu Ala Gly Glu Lys Leu Phe Pro Tyr Leu Asp Pro Phe
370 375 380

Asp Ser Ala Ser Gln Leu Met Glu Pro Gly Arg Gln Ser Met Asp Leu
385 390 395 400

Val Glu Leu Val Ser Leu Phe Pro Thr Leu Ala Gly Leu Ala Gly Leu
405 410 415

Gln Val Pro Pro Arg Cys Pro Val Pro Ser Phe His Val Glu Leu Cys
420 425 430

Arg Glu Gly Lys Asn Leu Leu Lys His Phe Arg Phe Arg Asp Leu Glu
435 440 445

Glu Asp Pro Tyr Leu Pro Gly Asn Pro Arg Glu Leu Ile Ala Tyr Ser
450 455 460

Gln Tyr Pro Arg Pro Ser Asp Ile Pro Gln Trp Asn Ser Asp Lys Pro
465 470 475 480

[0006]

Ser Leu Lys Asp Ile Lys Ile Met Gly Tyr Ser Ile Arg Thr Ile Asp
485 490 495

Tyr Arg Tyr Thr Val Trp Val Gly Phe Asn Pro Asp Glu Phe Leu Ala
500 505 510

Asn Phe Ser Asp Ile His Ala Gly Glu Leu Tyr Phe Val Asp Ser Asp
515 520 525

Pro Leu Gln Asp His Asn Met Tyr Asn Asp Ser Gln Gly Gly Asp Leu
530 535 540

Phe Gln Leu Leu Met Pro
545 550

<210> 3
<211> 312
<212> PRT
<213> 智人

<400> 3

Met Pro Pro Pro Arg Thr Gly Arg Gly Leu Leu Trp Leu Gly Leu Val
1 5 10 15

Leu Ser Ser Val Cys Val Ala Leu Gly Ser Glu Thr Gln Ala Asn Ser
20 25 30

Thr Thr Asp Ala Leu Asn Val Leu Leu Ile Ile Val Asp Asp Leu Arg
35 40 45

Pro Ser Leu Gly Cys Tyr Gly Asp Lys Leu Val Arg Ser Pro Asn Ile
50 55 60

Asp Gln Leu Ala Ser His Ser Leu Leu Phe Gln Asn Ala Phe Ala Gln
65 70 75 80

Gln Ala Val Cys Ala Pro Ser Arg Val Ser Phe Leu Thr Gly Arg Arg
85 90 95

Pro Asp Thr Thr Arg Leu Tyr Asp Phe Asn Ser Tyr Trp Arg Val His
100 105 110

[0007]

Ala Gly Asn Phe Ser Thr Ile Pro Gln Tyr Phe Lys Glu Asn Gly Tyr
115 120 125

Val Thr Met Ser Val Gly Lys Val Phe His Pro Gly Ile Ser Ser Asn
130 135 140

His Thr Asp Asp Ser Pro Tyr Ser Trp Ser Phe Pro Pro Tyr His Pro
145 150 155 160

Ser Ser Glu Lys Tyr Glu Asn Thr Lys Thr Cys Arg Gly Pro Asp Gly
165 170 175

Glu Leu His Ala Asn Leu Leu Cys Pro Val Asp Val Leu Asp Val Pro
180 185 190

Glu Gly Thr Leu Pro Asp Lys Gln Ser Thr Glu Gln Ala Ile Gln Leu
195 200 205

Leu Glu Lys Met Lys Thr Ser Ala Ser Pro Phe Phe Leu Ala Val Gly
210 215 220

Tyr His Lys Pro His Ile Pro Phe Arg Tyr Pro Lys Glu Phe Gln Lys
225 230 235 240

Leu Tyr Pro Leu Glu Asn Ile Thr Leu Ala Pro Asp Pro Glu Val Pro
245 250 255

Asp Gly Leu Pro Pro Val Ala Tyr Asn Pro Trp Met Asp Ile Arg Gln
260 265 270

Arg Glu Asp Val Gln Ala Leu Asn Ile Ser Val Pro Tyr Gly Pro Ile
275 280 285

Pro Val Asp Phe Gln Glu Asp Gln Ser Ser Thr Gly Phe Arg Leu Lys
290 295 300

Thr Ser Ser Thr Arg Lys Tyr Lys
305 310

<210> 4
<211> 343
<212> PRT

[0008]

<213> 智人

<400> 4

Met Pro Pro Pro Arg Thr Gly Arg Gly Leu Leu Trp Leu Gly Leu Val
1 5 10 15

Leu Ser Ser Val Cys Val Ala Leu Gly Ser Glu Thr Gln Ala Asn Ser
20 25 30

Thr Thr Asp Ala Leu Asn Val Leu Leu Ile Ile Val Asp Asp Leu Arg
35 40 45

Pro Ser Leu Gly Cys Tyr Gly Asp Lys Leu Val Arg Ser Pro Asn Ile
50 55 60

Asp Gln Leu Ala Ser His Ser Leu Leu Phe Gln Asn Ala Phe Ala Gln
65 70 75 80

Gln Ala Val Cys Ala Pro Ser Arg Val Ser Phe Leu Thr Gly Arg Arg
85 90 95

Pro Asp Thr Thr Arg Leu Tyr Asp Phe Asn Ser Tyr Trp Arg Val His
100 105 110

Ala Gly Asn Phe Ser Thr Ile Pro Gln Tyr Phe Lys Glu Asn Gly Tyr
115 120 125

Val Thr Met Ser Val Gly Lys Val Phe His Pro Gly Ile Ser Ser Asn
130 135 140

His Thr Asp Asp Ser Pro Tyr Ser Trp Ser Phe Pro Pro Tyr His Pro
145 150 155 160

Ser Ser Glu Lys Tyr Glu Asn Thr Lys Thr Cys Arg Gly Pro Asp Gly
165 170 175

Glu Leu His Ala Asn Leu Leu Cys Pro Val Asp Val Leu Asp Val Pro
180 185 190

Glu Gly Thr Leu Pro Asp Lys Gln Ser Thr Glu Gln Ala Ile Gln Leu
195 200 205

[0009]

Leu Glu Lys Met Lys Thr Ser Ala Ser Pro Phe Phe Leu Ala Val Gly
210 215 220

Tyr His Lys Pro His Ile Pro Phe Arg Tyr Pro Lys Glu Phe Gln Lys
225 230 235 240

Leu Tyr Pro Leu Glu Asn Ile Thr Leu Ala Pro Asp Pro Glu Val Pro
245 250 255

Asp Gly Leu Pro Pro Val Ala Tyr Asn Pro Trp Met Asp Ile Arg Gln
260 265 270

Arg Glu Asp Val Gln Ala Leu Asn Ile Ser Val Pro Tyr Gly Pro Ile
275 280 285

Pro Val Asp Phe Gln Arg Lys Ile Arg Gln Ser Tyr Phe Ala Ser Val
290 295 300

Ser Tyr Leu Asp Thr Gln Val Gly Arg Leu Leu Ser Ala Leu Asp Asp
305 310 315 320

Leu Gln Leu Ala Asn Ser Thr Ile Ile Ala Phe Thr Ser Asp His Gly
325 330 335

Phe Leu Met Arg Thr Asn Thr
340

<210> 5
<211> 341
<212> PRT
<213> 智人

<400> 5

Ser Gln Glu Ala Gly Thr Gly Ala Gly Ala Gly Ser Leu Ala Gly Ser
1 5 10 15

Cys Gly Cys Gly Thr Pro Gln Arg Pro Gly Ala His Gly Ser Ser Ala
20 25 30

Ala Ala His Arg Tyr Ser Arg Glu Ala Asn Ala Pro Gly Pro Val Pro
35 40 45

Gly Glu Arg Gln Leu Ala His Ser Lys Met Val Pro Ile Pro Ala Gly

[0010]

50 55 60

Val Phe Thr Met Gly Thr Asp Asp Pro Gln Ile Lys Gln Asp Gly Glu
65 70 75 80

Ala Pro Ala Arg Arg Val Thr Ile Asp Ala Phe Tyr Met Asp Ala Tyr
85 90 95

Glu Val Ser Asn Thr Glu Phe Glu Lys Phe Val Asn Ser Thr Gly Tyr
100 105 110

Leu Thr Glu Ala Glu Lys Phe Gly Asp Ser Phe Val Phe Glu Gly Met
115 120 125

Leu Ser Glu Gln Val Lys Thr Asn Ile Gln Gln Ala Val Ala Ala Ala
130 135 140

Pro Trp Trp Leu Pro Val Lys Gly Ala Asn Trp Arg His Pro Glu Gly
145 150 155 160

Pro Asp Ser Thr Ile Leu His Arg Pro Asp His Pro Val Leu His Val
165 170 175

Ser Trp Asn Asp Ala Val Ala Tyr Cys Thr Trp Ala Gly Lys Arg Leu
180 185 190

Pro Thr Glu Ala Glu Trp Glu Tyr Ser Cys Arg Gly Gly Leu His Asn
195 200 205

Arg Leu Phe Pro Trp Gly Asn Lys Leu Gln Pro Lys Gly Gln His Tyr
210 215 220

Ala Asn Ile Trp Gln Gly Glu Phe Pro Val Thr Asn Thr Gly Glu Asp
225 230 235 240

Gly Phe Gln Gly Thr Ala Pro Val Asp Ala Phe Pro Pro Asn Gly Tyr
245 250 255

Gly Leu Tyr Asn Ile Val Gly Asn Ala Trp Glu Trp Thr Ser Asp Trp
260 265 270

Trp Thr Val His His Ser Val Glu Glu Thr Leu Asn Pro Lys Gly Pro

[0011]

275 280 285

Pro Ser Gly Lys Asp Arg Val Lys Lys Gly Gly Ser Tyr Met Cys His
290 295 300

Arg Ser Tyr Cys Tyr Arg Tyr Arg Cys Ala Ala Arg Ser Gln Asn Thr
305 310 315 320

Pro Asp Ser Ser Ala Ser Asn Leu Gly Phe Arg Cys Ala Ala Asp Arg
325 330 335

Leu Pro Thr Met Asp
340

<210> 6
<211> 374
<212> PRT
<213> 智人

<400> 6

Met Ala Ala Pro Ala Leu Gly Leu Val Cys Gly Arg Cys Pro Glu Leu
1 5 10 15

Gly Leu Val Leu Leu Leu Leu Leu Leu Ser Leu Leu Cys Gly Ala Ala
20 25 30

Gly Ser Gln Glu Ala Gly Thr Gly Ala Gly Ala Gly Ser Leu Ala Gly
35 40 45

Ser Cys Gly Cys Gly Thr Pro Gln Arg Pro Gly Ala His Gly Ser Ser
50 55 60

Ala Ala Ala His Arg Tyr Ser Arg Glu Ala Asn Ala Pro Gly Pro Val
65 70 75 80

Pro Gly Glu Arg Gln Leu Ala His Ser Lys Met Val Pro Ile Pro Ala
85 90 95

Gly Val Phe Thr Met Gly Thr Asp Asp Pro Gln Ile Lys Gln Asp Gly
100 105 110

Glu Ala Pro Ala Arg Arg Val Thr Ile Asp Ala Phe Tyr Met Asp Ala
115 120 125

[0012]

Tyr Glu Val Ser Asn Thr Glu Phe Glu Lys Phe Val Asn Ser Thr Gly
130 135 140

Tyr Leu Thr Glu Ala Glu Lys Phe Gly Asp Ser Phe Val Phe Glu Gly
145 150 155 160

Met Leu Ser Glu Gln Val Lys Thr Asn Ile Gln Gln Ala Val Ala Ala
165 170 175

Ala Pro Trp Trp Leu Pro Val Lys Gly Ala Asn Trp Arg His Pro Glu
180 185 190

Gly Pro Asp Ser Thr Ile Leu His Arg Pro Asp His Pro Val Leu His
195 200 205

Val Ser Trp Asn Asp Ala Val Ala Tyr Cys Thr Trp Ala Gly Lys Arg
210 215 220

Leu Pro Thr Glu Ala Glu Trp Glu Tyr Ser Cys Arg Gly Gly Leu His
225 230 235 240

Asn Arg Leu Phe Pro Trp Gly Asn Lys Leu Gln Pro Lys Gly Gln His
245 250 255

Tyr Ala Asn Ile Trp Gln Gly Glu Phe Pro Val Thr Asn Thr Gly Glu
260 265 270

Asp Gly Phe Gln Gly Thr Ala Pro Val Asp Ala Phe Pro Pro Asn Gly
275 280 285

Tyr Gly Leu Tyr Asn Ile Val Gly Asn Ala Trp Glu Trp Thr Ser Asp
290 295 300

Trp Trp Thr Val His His Ser Val Glu Glu Thr Leu Asn Pro Lys Gly
305 310 315 320

Pro Pro Ser Gly Lys Asp Arg Val Lys Lys Gly Gly Ser Tyr Met Cys
325 330 335

His Arg Ser Tyr Cys Tyr Arg Tyr Arg Cys Ala Ala Arg Ser Gln Asn
340 345 350

[0013]

Thr Pro Asp Ser Ser Ala Ser Asn Leu Gly Phe Arg Cys Ala Ala Asp
355 360 365

Arg Leu Pro Thr Met Asp
370

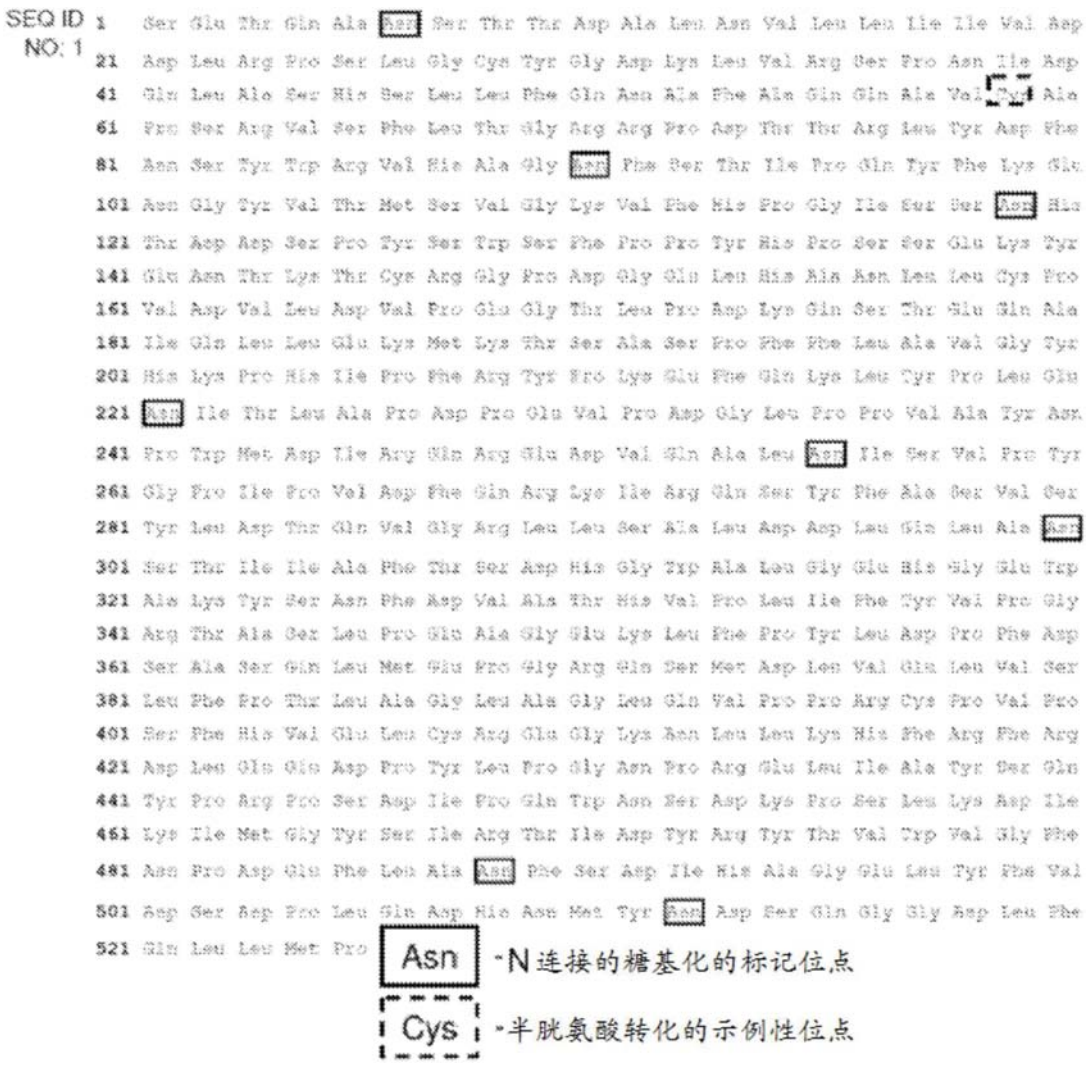

SEQ ID NO: 1
1 Ser Glu Thr Gln Ala Asn Ser Thr Thr Asp Ala Leu Asn Val Leu Leu Ile Ile Val Asp
21 Asp Leu Arg Pro Ser Leu Gly Cys Tyr Gly Asp Lys Leu Val Arg Ser Pro Asn Ile Asp
41 Gln Leu Ala Ser His Ser Leu Leu Phe Gln Asn Ala Phe Ala Gln Gln Ala Val Cys Ala
61 Pro Ser Arg Val Ser Phe Leu Thr Gly Arg Arg Pro Asp Thr Thr Arg Leu Tyr Asp Phe
81 Asn Ser Tyr Trp Arg Val His Ala Gly Asn Phe Ser Thr Ile Pro Gln Tyr Phe Lys Glu
101 Asn Gly Tyr Val Thr Met Ser Val Gly Lys Val Phe His Pro Gly Ile Ser Ser Asn His
121 Thr Asp Asp Ser Pro Tyr Ser Trp Ser Phe Pro Pro Tyr His Pro Ser Ser Glu Lys Tyr
141 Glu Asn Thr Lys Thr Cys Arg Gly Pro Asp Gly Glu Leu His Ala Asn Leu Leu Cys Pro
161 Val Asp Val Leu Asp Val Pro Glu Gly Thr Leu Pro Asp Lys Gln Ser Thr Glu Gln Ala
181 Ile Gln Leu Leu Glu Lys Met Lys Thr Ser Ala Ser Pro Phe Phe Leu Ala Val Gly Tyr
201 His Lys Pro His Ile Pro Phe Arg Tyr Pro Lys Glu Phe Gln Lys Leu Tyr Pro Leu Glu
221 Asn Ile Thr Leu Ala Pro Asp Pro Glu Val Pro Asp Gly Leu Pro Pro Val Ala Tyr Asn
241 Pro Trp Met Asp Ile Arg Gln Arg Glu Asp Val Gln Ala Leu Asn Ile Ser Val Pro Tyr
261 Gly Pro Ile Pro Val Asp Phe Gln Arg Lys Ile Arg Gln Ser Tyr Phe Ala Ser Val Ser
281 Tyr Leu Asp Thr Gln Val Gly Arg Leu Leu Ser Ala Leu Asp Asp Leu Gln Leu Ala Asn
301 Ser Thr Ile Ile Ala Phe Thr Ser Asp His Gly Trp Ala Leu Gly Glu His Gly Glu Trp
321 Ala Lys Tyr Ser Asn Phe Asp Val Ala Thr His Val Pro Leu Ile Phe Tyr Val Pro Gly
341 Arg Thr Ala Ser Leu Pro Glu Ala Gly Glu Lys Leu Phe Pro Tyr Leu Asp Pro Phe Asp
361 Ser Ala Ser Gln Leu Met Glu Pro Gly Arg Gln Ser Met Asp Leu Val Glu Leu Val Ser
381 Leu Phe Pro Thr Leu Ala Gly Leu Ala Gly Leu Gln Val Pro Pro Arg Cys Pro Val Pro
401 Ser Phe His Val Glu Leu Cys Arg Glu Gly Lys Asn Leu Leu Lys His Phe Arg Phe Arg
421 Asp Leu Glu Glu Asp Pro Tyr Leu Pro Gly Asn Pro Arg Glu Leu Ile Ala Tyr Ser Gln
441 Tyr Pro Arg Pro Ser Asp Ile Pro Gln Trp Asn Ser Asp Lys Pro Ser Leu Lys Asp Ile
461 Lys Ile Met Gly Tyr Ser Ile Arg Thr Ile Asp Tyr Arg Tyr Thr Val Trp Val Gly Phe
481 Asn Pro Asp Glu Phe Leu Ala Asn Phe Ser Asp Ile His Ala Gly Glu Leu Tyr Phe Val
501 Asp Ser Asp Pro Leu Gln Asp His Asn Met Tyr Asn Asp Ser Gln Gly Gly Asp Leu Phe
521 Gln Leu Leu Met Pro
Asn -N连接的糖基化的标记位点
Cys -半胱氨酸转化的示例性位点

图 1

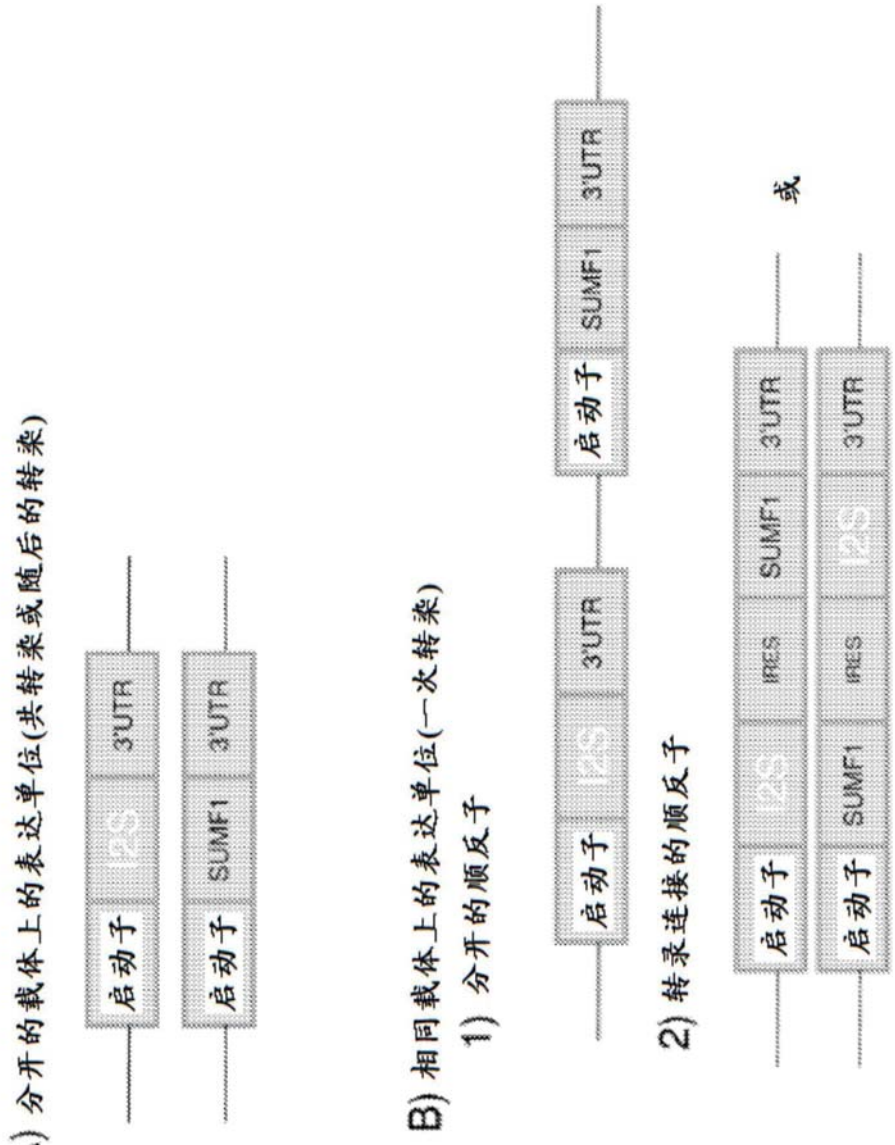
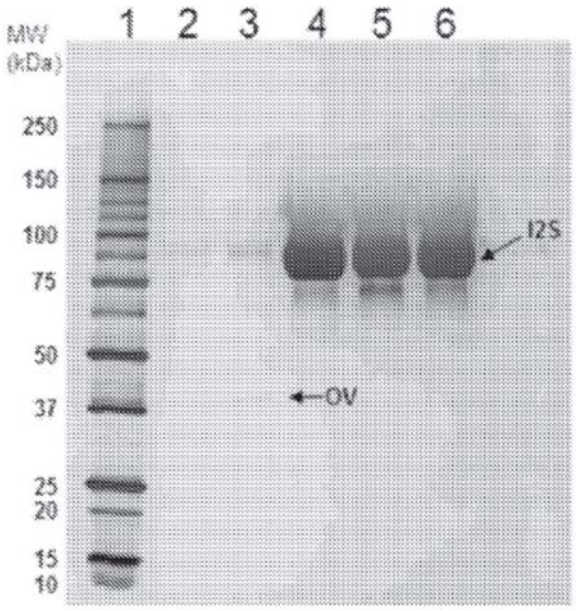

I2S 和 SUMF1 共表达选择
A) 分开的载体上的表达单位(共转染或随后的转染)
启动子
I2S
3'UTR
启动子
SUMF1
3'UTR
B) 相同载体上的表达单位(一次转染)
1) 分开的顺反子
启动子
I2S
3'UTR
启动子
SUMF1
3'UTR
2) 转录连接的顺反子
启动子
I2S
IRES
SUMF1
3'UTR
或
启动子
SUMF1
IRES
I2S
3'UTR

图 2



泳道	上样样品	上样体积	总计 µg
1	蛋白质标准	3 µl	
2	测定对照 #1	20 µl	8 µg
3	测定对照 #2	20 µl	16 µg
4	I2S 参考标准	20 µl	8 µg
5	I2S-AF 2D 无血清培养物	20 µl	8 µg
6	I2S-AF 4D 无血清培养物	20 µl	8 µg

图 3

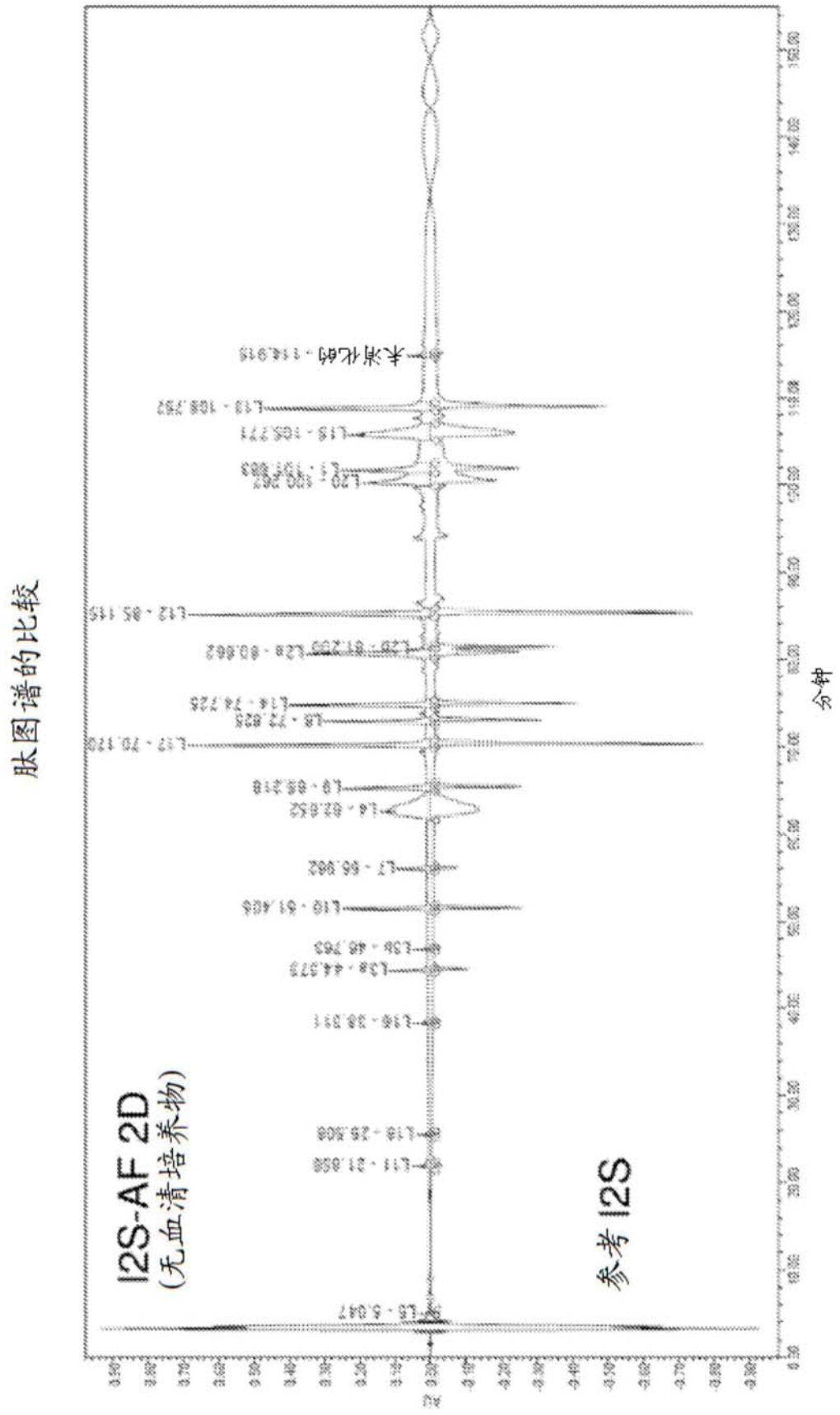


图 4

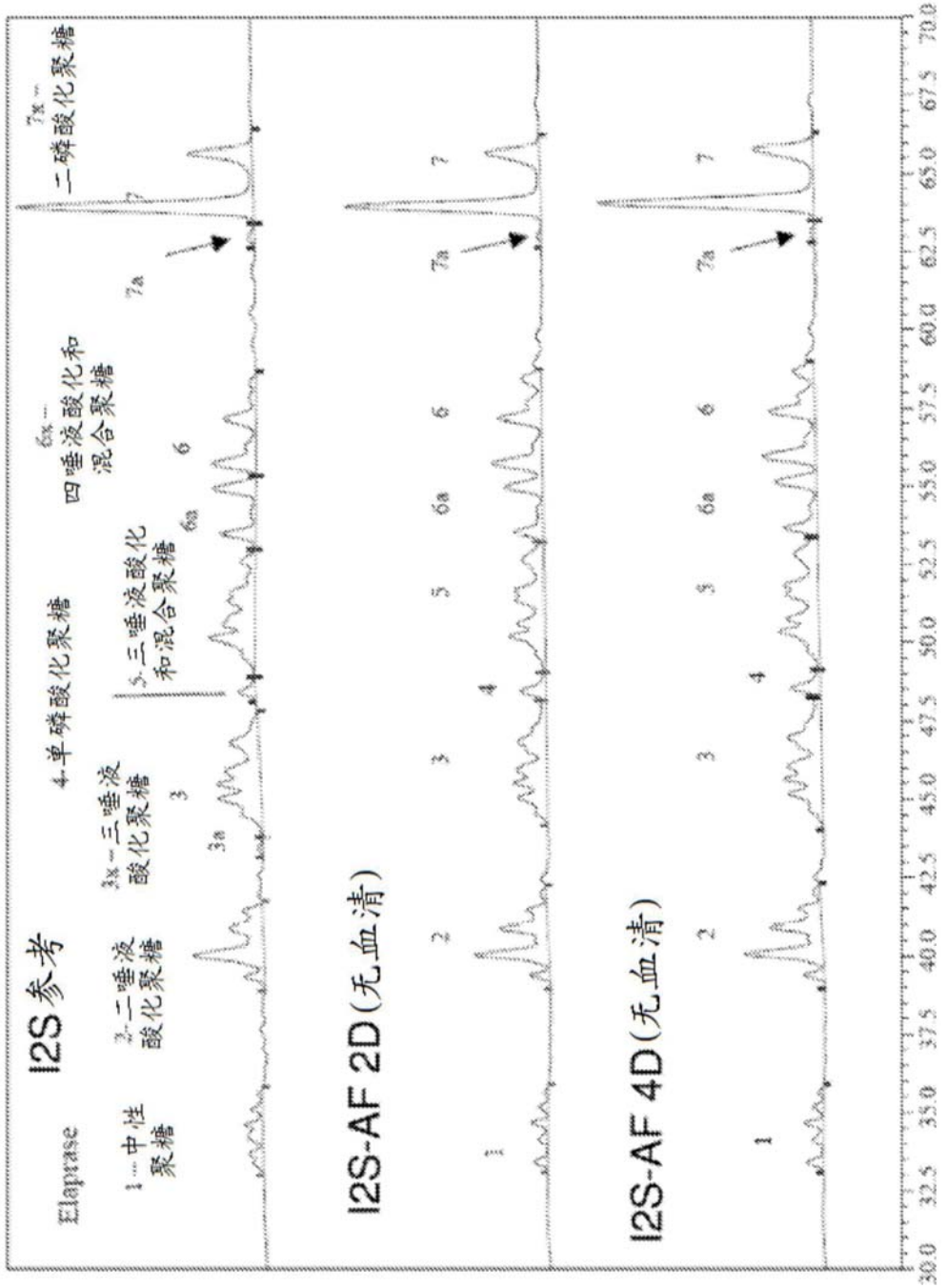

I2S 参考
Elaprase
1…中性聚糖
2…二唾液酸化聚糖
3x…三唾液酸化聚糖
4…单磷酸化聚糖
5…三唾液酸化和混合聚糖
6x…四唾液酸化和混合聚糖
7x…二磷酸化聚糖
I2S-AF 2D(无血清)
I2S-AF 4D(无血清)
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
70.0

图 5

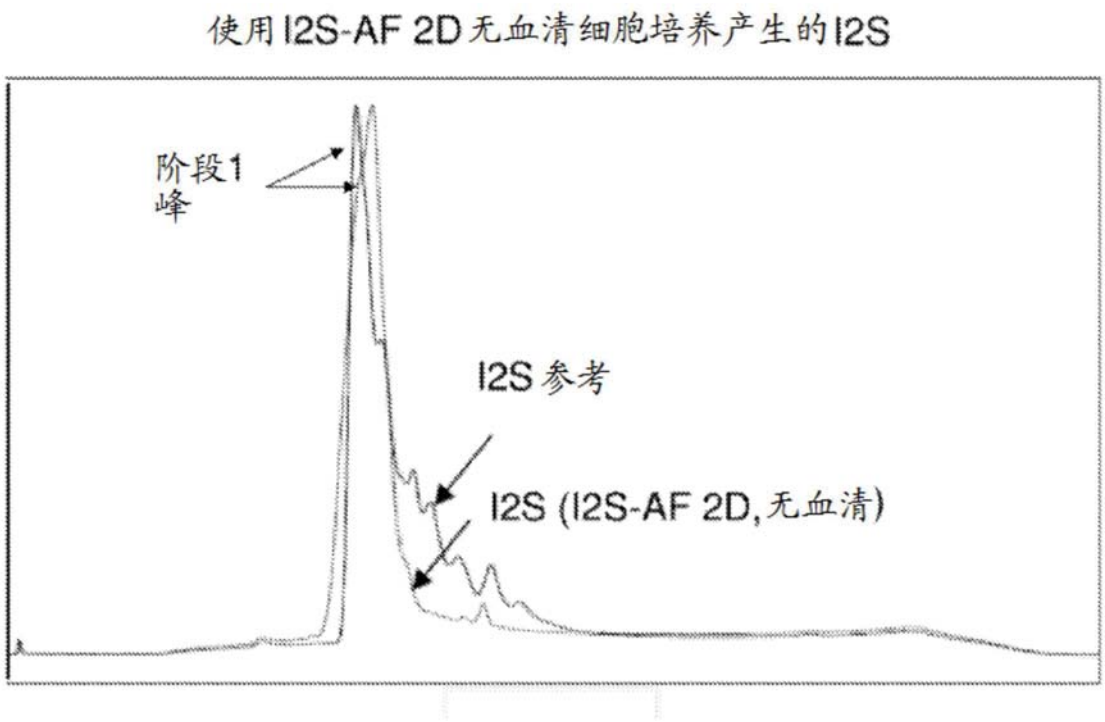


图 6

Abstract

The present invention provides, among other things, methods and compositions for large-scale production of recombinant I2S protein using suspension culture of mammalian cells in serum-free medium. In particular, the present invention uses mammalian cells co-express a recombinant I2S protein and a formylglycine generating enzyme (FGE).