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(54) Title: PURIFICATION OF IDURONATE-2-SULFATASE

(57) Abstract: The present invention provides, among other things, improved methods for purifying 12 S protein produced recombinantly for enzyme replacement therapy. The present invention is, in part, based on the surprising discovery that recombinant I2S protein can be purified from unprocessed biological materials, such as, I2S-containing cell culture medium, using a process involving as few as four chromatography columns.

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PURIFICATION OF IDURONATE-2-SULFATASE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 61/666,733, 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 “2006685-0342_SEQ_LIST” on June 27, 2013. The .txt file was generated on June 25, 2013 and is 15 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, improved methods for purifying I2S protein produced recombinantly for enzyme replacement therapy. The present invention is, in part, based on the surprising discovery that recombinant I2S protein can be purified from unprocessed biological materials, such as, I2S-containing cell culture medium, using a process involving as few as four chromatography columns. Approved existing purification process of recombinant I2S for enzyme replacement therapy involves 6 chromatography columns. As described in the Examples section, recombinant I2S proteins purified using a four-column process according to the invention conforms with the marketing purity requirements in the US and many other countries. In addition, the recombinant I2S enzyme purified according to the present invention retains high percentage of C_α-formylglycine (FGly) (e.g., higher than 70% and up to 100%), which is important for the activity of I2S enzyme, and distinct characteristics such as sialic acid content and glycan map that may facilitate bioavailability and/or lysosomal targeting of the recombinant I2S protein. Therefore, the present invention provides an effective, cheaper, and faster process for purifying recombinant I2S protein. The present invention is particularly useful for purifying recombinant I2S protein produced in serum-free medium.

[0007] Thus, in one aspect, the present invention provides a method of purifying recombinant I2S protein from an impure preparation using a process based on one or more of anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography. In some embodiments, an inventive method according to the present invention involves less than 6 (e.g., less than 5, less than 4, or less than 3) chromatography steps. In some embodiments, an inventive method according to the present invention involves 2, 3, 4 or 5 chromatography steps. In some embodiments, an inventive method according to the present invention involves 4 chromatography steps. In some embodiments, the purified recombinant I2S protein according to the present invention contains less than 100 ng/mg Host Cell Protein (HCP) (e.g., less than 90 ng/mg HCP, less than 80 ng/mg HCP, less than 70 ng/mg HCP, less than 60 ng/mg HCP, less than 50 ng/mg HCP, less than 40 ng/mg HCP, less than 30 ng/mg HCP, less than 20 ng/mg HCP, less than 10 ng/mg HCP).

[0008] In some embodiments, a suitable anion-exchange chromatography is Q chromatography. In some embodiments, a suitable cation-exchange chromatography is SP chromatography. In some embodiments, a suitable mixed-mode chromatography is hydroxyapatite (HA) chromatography. In some embodiments, a suitable hydrophobic interaction chromatography is phenyl chromatography.

[0009] It is contemplated that anion-exchange chromatography (e.g., O column), cation-exchange chromatography (e.g., SP column), mixed-mode chromatography (e.g., HA column), and hydrophobic interaction chromatography (e.g., phenyl column) can be carried out in any order. In some embodiments, a method according to the present invention carries out anion-exchange chromatography (e.g., O column), cation-exchange chromatography (e.g., SP column), mixed-mode chromatography (e.g., HA column), and hydrophobic interaction chromatography (e.g., phenyl column) in that order.

[0010] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to pH of about 5.0-7.0 (e.g., about 5.0, 5.5, 6.0, 6.5 or 7.0) and the conductivity of about 10-20 mS/cm (e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 mS/cm) prior to loading to the anion-exchange chromatography column (e.g., Q column). In some embodiments, the pH is adjusted using 1M sodium acetate. In some embodiments, the conductivity is adjusted using 5 M sodium chloride. In some embodiments, the anion-exchange chromatography column, once loaded, is washed using a wash buffer comprising salt (e.g., NaCl) concentration ranging from about 140 mM to 200 mM (e.g., about 140 mM, 145 mM, 150 mM, 155 mM, 160 mM, 165 mM, 170 mM, 175 mM, 180 mM, 185 mM, 190 mM, 195 mM, or 200 mM) with pH of about 5.0-7.0 (e.g., about 5.0, 5.5, 6.0, 6.5 or 7.0). In some embodiments, the anion-exchange chromatography column is eluted using a elution buffer comprising a linear salt (e.g., NaCl) gradient. In some embodiments, a suitable linear NaCl gradient contains a range from about 0-500 mM NaCl (e.g., about 0-400 mM, about 0-

350 mM, about 0-300 mM, about 50-500 mM, about 150-500 mM, about 150-450 mM, about 150-400 mM).

[0011] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to conductivity ranging between about 1 mS/cm and 20 mS/cm (e.g., between about 1 mS/cm and 15 mS/cm, between about 1 mS/cm and 10 mS/cm, between about 1 mS/cm and 8 mS/cm, between about 1 mS/cm and 6 mS/cm, between about 1 mS/cm and 4 mS/cm, between about 2 mS/cm and 4 mS/cm) prior to loading to the cation-exchange chromatography column (e.g., SP column). In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to conductivity ranging between n about 2 mS/cm and 4 mS/cm (e.g., 2, 2.5, 3, 3.5, or 4 mS/cm) prior to loading to the cation-exchange chromatography column (e.g., SP column). In some embodiments, the conductivity is adjusted by diluting the eluate from the anion-exchange chromatography column with H_2O at about 1-2:1 (e.g.,. 1:1, 1.1:1, 1.2:1, 1.3:1, 1.4:, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, or 2:1) ratio. In some embodiments, the conductivity is adjusted by dialfiltration. In some embodiments, the cation-exchange chromatography column is run at a pH of about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0 or 6.5). In some embodiments, the cation-exchange chromatography column is run with a buffer comprising phosphate (e.g., NaPO4) concentration ranging from about 0.01 M to about 0.1 M (e.g., about 0.01 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M, 0.06 M, 0.07 M, 0.08 M, 0.09 M, or 0.1 M). In some embodiments, a suitable pH is about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0, or 6.5).

[0012] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to phosphate (e.g., NaPO4) concentration ranging from about 0.001 M to about 0.01 M (e.g., about 0.001 M, 0.002 M, 0.003 M, 0.004 M, 0.005 M, 0.006 M, 0.007 M, 0.008 M, 0.009 M, or 0.01 M) and pH of about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0, or 6.5) prior to loading the mixed-mode chromatography column (e.g., HA column). In some embodiments, the mixed-mode chromatography column (e.g., HA column), once loaded, is washed with a wash buffer containing phosphate (e.g., 1-10 mM sodium or potassium phosphate) at or near neutral pH. In some embodiments, the loaded mixed-mode chromatography column (e.g., HA column) is washed with a wash buffer having a phosphate concentration ranging from about 10-20 mM (e.g., about 10-18 mM, 10-16 mM, 10-15 mM, 12-20 mM, 14-18 mM, 14-16 mM). In some embodiments, the loaded mixed-mode chromatography column (e.g., HA column) is washed with a wash buffer having a phosphate concentration of or greater than 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM. In some embodiments, elution from a mixed-mode chromatography column (e.g., HA column) is achieved with a gradient phosphate buffer. In some embodiments, a suitable elution buffer may have a phosphate gradient of approximately 1-400 mM (e.g., 1-300 mM, 1-200 mM, 1-150 mM, 1-100 mM, 10-350 mM, 10-300 mM, 10-250 mM, 10-200 mM, 10-150 mM, 10-140 mM, 10-130 mM, 10-120 mM, 10-110 mM, 10-100 mM, 10-90 mM, 10-80 mM, 10-70 mM, 10-60 mM, 10-50 mM) sodium phosphate or potassium phosphate. In some embodiments, elution from an HA column is achieved by stepwise increasing the phosphate concentration in the elution buffer. In some embodiments, stepwise elution buffers may have a phosphate (e.g., sodium phosphate) concentration selected from 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM. In some embodiments, elution from a mixed-mode chromatography column (e.g., HA column) is achieved by an elution buffer having a phosphate (e.g., sodium phosphate) concentration ranging from about 50 mM to 150 mM (e.g., selected from the phosphate (e.g., sodium phosphate) concentration of 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, and combination thereof).

[0013] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to salt (e.g., NaCl) concentration ranging from about 0.5 M to about 2.0 M (e.g., about 0.5 M, 1.0 M, 1.1 M, 1.2 M, 1.3 M, 1.4 M, 1.5 M, 1.6 M, 1.7 M, 1.8 M, 1.9 M, or 2.0 M NaCl) at pH of about 4.5-6.0 (e.g., about 4.5, 5.0, 5.5, or 6.0) prior to loading onto the hydrophobic interaction chromatography column (e.g., phenyl column). In some embodiments, the hydrophobic interaction chromatography column, once loaded, is washed using a wash buffer comprising salt (e.g., NaCl) concentration ranging from about 0.5 M to 2.0 M (e.g., about 0.5 M, 1.0 M, 1.1 M, 1.2 M, 1.3 M, 1.4 M, 1.5 M, 1.6 M, 1.7 M, 1.8 M, 1.9 M, or 2.0 M NaCl) at pH of about 4.5-6.0 (e.g., about 4.5, 5.0, 5.5, or 6.0). In some embodiments, the hydrophobic interaction chromatography column is eluted using a elution buffer comprising salt (e.g., NaCl) concentration ranging from about 0.1 M to about 0.5 M (e.g., about 0.1 M, 0.2 M, 0.3 M, 0.4 M, or 0.5 M NaCl) at pH of about 4.5-6.0 (e.g., about 4.5, 5.0, 5.5, or 6.0).

[0014] In some embodiments, each of the anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography column has a height ranging from 14-25 cm (e.g., 15-25 cm, 15-20 cm, 14-24 cm, 14-22 cm, 14-20 cm, or 16-18 cm). In some embodiments, each of the anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography column has a height of approximately 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 cm.

[0015] In some embodiments, an inventive method according to the present invention includes a step of viral inactivation before loading the impure preparation onto the first chromatography column. In some embodiments, the step of viral inactivation includes adding a detergent to the impure preparation. In some embodiments, an inventive method according to the invention further includes a step of viral removal after the last Chromatography column. In some embodiments, a method of the invention further includes a step of ultrafiltration and/or dialfiltration. In some embodiments, the step of ultrafiltration and/or dialfiltration includes exchanging the purified recombinant I2S protein into a drug formulation buffer.

[0016] In some embodiments, the present invention is used to purify 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, the present invention is used to purify a recombinant I2S protein having an amino acid sequence identical to SEQ ID NO:1.

[0017] In some embodiments, the present invention is used to purify a recombinant I2S protein produced by mammalian cells cultured in suspension in a serum-free medium. In some embodiments, a serum-free medium suitable for the invention lacks animal-derived components. In some embodiments, a serum-free medium suitable for the invention is a chemically-defined medium. In some embodiments, the mammalian cells are cultured in a bioreactor. In some embodiments, the mammalian cells co-express the recombinant I2S protein and formylglycine generating enzyme (FGE). In some embodiments, the mammalian cells are human cells.

[0018] In some embodiments, an impure preparation used in a method of the invention is prepared from the serum-free medium containing recombinant I2S protein secreted from the mammalian cells. In some embodiments, an impure preparation used in a method of the invention is thawed from a frozen medium preparation.

[0019] In some embodiments, a purified recombinant I2S protein according to the present invention contains, on average, 16-22 (e.g., 16-21, 16-20, 16-19, 17-22, 17-21, 17-20, 17-19) sialic acids per molecule. In some embodiments, a purified recombinant I2S protein according to the present invention contains, on average, 16, 17, 18, 19, 20, 21, or 22 sialic acids per molecule.

[0020] In some embodiments, a purified recombinant I2S protein according to the present invention 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, a purified recombinant I2S protein according to the present invention 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, a purified recombinant I2S protein according to the present invention has specific activity of at least 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 as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

[0021] In some embodiments, a purified recombinant I2S protein according to the present invention is characterized with cellular uptake of greater than 70%, 75%, 80%, 85%, 90%, 95%, as determined by an *in vitro* uptake assay.

[0022] In some embodiments, a purified recombinant I2S protein according to the present invention is characterized with a glycan map comprising seven peak groups indicative of neutral (peak group 1), mono-sialylated (peak group 2), di-sialylated (peak group 3), monophosphorylated (peak group 4), tri-sialylated (peak group 5), tetra-sialylated (peak group 6), and diphosphorylated (peak group 7) I2S protein, respectively. In some embodiments, the glycan map is generated following a neuraminidase digestion. In other embodiments, the glycan map is generated following an alkaline phosphatase digestion.

[0023] Among other things, the present invention provides purified recombinant I2S protein as described herein, and pharmaceutical compositions or formulation containing the same. In some embodiments, a formulation is formulated for intravenous, subcutaneous and/or intrathecal administration. The present invention also provides methods of treating Hunter syndrome by administering into a subject in need of treatment a purified recombinant I2S, pharmaceutical composition or formulation containing the same.

[0024] 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.

[0025] 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.

[0026] 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 DRAWING

[0027] The Figures described below, that together make up the Drawing, are for illustration purposes only, not for limitation.

[0028] *Figure 1* depicts an exemplary purification scheme for recombinant I2S produced in serum free medium.

[0029] *Figure 2* depicts an exemplary peptide maps of purified recombinant I2S AF as compared to a reference I2S.

[0030] *Figure 3* depicts an exemplary SDS-PAGE (Silver) analysis of purified recombinant I2S AF.

[0031] *Figure 4* depicts an exemplary charge profile analysis of purified recombinant I2S AF assessed by ion-exchange chromatography.

[0032] *Figure 5* depicts exemplary glycan map profiles of purified recombinant I2S AF.

[0033] *Figure 6* depicts an exemplary analysis of activity (U/mg) after a viral inactivation UPB step of a clarified harvest of recombinant I2S.

[0034] *Figure 7* depicts an exemplary analysis of SEC-HPLC after a viral inactivation UPB step of a clarified harvest of recombinant I2S.

[0035] *Figure 8* depicts exemplary SDS-PAGE treated with silver stain of purified recombinant I2S protein.

[0036] *Figure 9* shows an exemplary peptide map for a purified recombinant I2S enzyme produced from the I2S-AF 2D cell line grown under serum-free culture conditions (top panel) as compared to a reference.

[0037] *Figure 10* depicts exemplary glycan profiles generated for purified recombinant I2S enzymes produced using the I2S-AF 2D and 4D cell lines grown under serum-free cell culture conditions as compared to a reference.

[0038] *Figure 11* depicts an exemplary charge profile generated for purified recombinant I2S enzyme produced using the I2S-AF 2D cell line grown under serum-free cell culture conditions as compared to an I2S reference control.

DEFINITIONS

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

[0040] *Approximately or about:* 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).

[0041] *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.

[0042] *Cation-independent mannose-6-phosphate receptor (CI-MPR):* As used herein, the term "cation-independent mannose-6-phosphate receptor (CI-MPR)" refers to a cellular receptor that binds mannose-6-phosphate (M6P) tags on acid hydrolase precursors in the Golgi apparatus that are destined for transport to the lysosome. In addition to mannose-6-phosphates, the CI-MPR also binds other proteins including IGF-II. The CI-MPR is also known as "M6P/IGF-II receptor," "CI-MPR/IGF-II receptor," "IGF-II receptor" or "IGF2 Receptor." These terms and abbreviations thereof are used interchangeably herein.

[0043] *Chromatography:* As used herein, the term "chromatography" refers to a technique for separation of mixtures. Typically, the mixture is dissolved in a fluid called the "mobile phase," which carries it through a structure holding another material called the "stationary phase." Column chromatography is a separation technique in which the stationary bed is within a tube, i.e., column.

[0044] *Diluent*: As used herein, the term "diluent" refers to a pharmaceutically acceptable (e.g., safe and non-toxic for administration to a human) diluting substance useful for the preparation of a reconstituted formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0045] *Elution*: As used herein, the term "elution" refers to the process of extracting one material from another by washing with a solvent. For example, in ion-exchange chromatography, elution is a process to wash loaded resins to remove captured ions.

[0046] *Eluate*: As used herein, the term "eluate" refers to a combination of mobile phase "carrier" and the analyte material that emerge from the chromatography, typically as a result of eluting.

[0047] *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. 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.

[0048] *Equilibrate or Equilibration:* As used herein, the terms "equilibrate" or "equilibration" in relation to chromatography refer to the process of bringing a first liquid (e.g., buffer) into balance with another, generally to achieve a stable and equal distribution of components of the liquid (e.g., buffer). For example, in some embodiments, a chromatographic column may be equilibrated by passing one or more column volumes of a desired liquid (e.g., buffer) through the column.

[0049] *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).

[0050] *Impurities*: As used herein, the term "impurities" refers to substances inside a confined amount of liquid, gas, or solid, which differ from the chemical composition of the target material or compound. Impurities are also referred to as contaminants.

[0051] *Linker:* As used herein, the term "linker" refers to, in a fusion protein, an amino acid sequence other than that appearing at a particular position in the natural protein and is generally designed to be flexible or to interpose a structure, such as an a-helix, between two protein moieties. A linker is also referred to as a spacer.

[0052] *Load:* As used herein, the term "load" refers to, in chromatography, adding a sample-containing liquid or solid to a column. In some embodiments, particular components of the sample loaded onto the column are then captured as the loaded sample passes through the column. In some embodiments, particular components of the sample loaded onto the column are not captured by, or "flow through", the column as the loaded sample passes through the column.

[0053] *Polypeptide*: As used herein, a "polypeptide", generally speaking, is a string of at least two amino acids attached to one another by a peptide bond. In some embodiments, a polypeptide may include at least 3-5 amino acids, each of which is attached to others by way of at least one peptide bond. Those of ordinary skill in the art will appreciate that polypeptides sometimes include "non-natural" amino acids or other entities that nonetheless are capable of integrating into a polypeptide chain, optionally.

[0054] *Pool:* As used herein, the term "pool" in relation to chromatography refers to combining one or more fractions of fluid that has passed through a column together. For example, in some embodiments, one or more fractions which contain a desired component of a sample that has been separated by chromatography (e.g., "peak fractions") can be "pooled" together generate a single "pooled" fraction.

[0055] *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.

[0056] *Soluble:* As used herein, the term "soluble" refers to the ability of a therapeutic agent to form a homogenous solution. In some embodiments, the solubility of the therapeutic agent in the solution into which it is administered and by which it is transported to the target site of action is sufficient to permit the delivery of a therapeutically effective amount of the therapeutic agent to the targeted site of action. Several factors can impact the solubility of the therapeutic agents. For example, relevant factors which may impact protein solubility include ionic strength, amino acid sequence and the presence of other co-solubilizing agents or salts (e.g., calcium salts). In some embodiments, therapeutic agents in accordance with the present invention are soluble in its corresponding pharmaceutical composition.

[0057] *Stability:* As used herein, the term "stable" refers to the ability of the therapeutic agent (e.g., a recombinant enzyme) to maintain its therapeutic efficacy (e.g., all or the majority of its intended biological activity and/or physiochemical integrity) over extended periods of time. The stability of a therapeutic agent, and the capability of the pharmaceutical composition to maintain stability of such therapeutic agent, may be assessed over extended periods of time (e.g., for at least 1, 3, 6, 12, 18, 24, 30, 36 months or more). In the context of a formulation a stable formulation is one in which the therapeutic agent therein essentially retains its physical and/or chemical integrity and biological activity upon storage and during processes (such as freeze/thaw, mechanical mixing and lyophilization). For protein stability, it can be measure by formation of high molecular weight (HMW) aggregates, loss of enzyme activity, generation of peptide fragments and shift of charge profiles.

[0058] *Viral Processing:* As used herein, the term "viral processing" refers to "viral removal," in which viruses are simply removed from the sample, or "viral inactivation," in which the viruses remain in a sample but in a non-infective form. In some embodiments, viral removal may utilize nanofiltration and/or chromatographic techniques, among others. In some embodiments, viral inactivation may utilize solvent inactivation, detergent inactivation, pasteurization, acidic pH inactivation, and/or ultraviolet inactivation, among others.

DETAILED DESCRIPTION OF THE INVENTION

[0059] The present invention provides, among other things, an improved method for purifying recombinant I2S protein for enzyme replacement therapy based on a process involving less than 6 chromatography steps. In some embodiments, the present invention provides a method of purifying recombinant I2S protein from an impure preparation using a process based on one or more of anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography. In some embodiments, the present invention provides a method of purifying recombinant I2S protein from an impure preparation by conducting Q chromatography, hydroxyapatite (HA) chromatography, SP chromatography, and phenyl chromatography. The present invention further provides purified recombinant I2S protein and method of use.

[0060] 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.

Recombinant I2S Protein

[0061] 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.

[0062] 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	SETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP(SEQ ID NO:1)
Full-Length Precursor (Isoform a)	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP(SEQ ID NO:2)
Isoform b Precursor	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQEDQSSTGFRLKTSSTRKYK (SEQ ID NO:3)
Isoform c Precursor	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGFLMRTNT(SEQ ID No:4)

[0063] Thus, in some embodiments, a recombinant I2S protein 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 recombinant I2S protein 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 recombinant I2S protein is substantially homologous to mature human I2S protein (SEQ ID NO:1). In some embodiments, a recombinant I2S protein 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 recombinant I2S protein is substantially identical to mature human I2S protein (SEQ ID NO:1). In some embodiments, a recombinant I2S protein 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 recombinant I2S protein contains a fragment or a portion of mature human I2S protein.

[0064] Alternatively, a recombinant I2S protein is full-length I2S protein. In some embodiments, a recombinant I2S protein 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, a recombinant I2S protein is substantially homologous to full-length human I2S protein (SEQ ID NO:2). For example, a recombinant I2S protein may have 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, a recombinant I2S protein is substantially identical to SEQ ID NO:2. For example, a recombinant I2S protein may have 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, a recombinant I2S protein 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.

[0065] In some embodiments, a recombinant I2S protein is human I2S isoform a protein. In some embodiments, a recombinant I2S protein 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, a recombinant I2S protein is substantially homologous to human I2S isoform a protein (SEQ ID NO:3). For example, a recombinant I2S protein may have 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, a recombinant I2S protein is substantially identical to SEQ ID NO:3. For example, a recombinant I2S protein may have 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, a recombinant I2S protein 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.

[0066] In some embodiments, a recombinant I2S protein is human I2S isoform b protein. In some embodiments, a recombinant I2S protein 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, a recombinant I2S protein is substantially homologous to human I2S isoform b protein (SEQ ID NO:4). For example, a recombinant I2S protein may have 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, a recombinant I2S protein is substantially identical to SEQ ID NO:4. For example, a recombinant I2S protein may have 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, a recombinant I2S protein 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.

[0067] 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.

[0068] In some embodiments, recombinant I2S proteins may contain a moiety that binds to a receptor on the surface of target 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. In some embodiments, a recombinant I2S protein contains M6P residues on the surface of the protein. In particular, a recombinant I2S protein may contain bis-phosphorylated oligosaccharides which have higher binding affinity to the CI-MPR. In some embodiments, a suitable enzyme contains up to about an average of about at least 20% bis-phosphorylated oligosaccharides per enzyme. In other embodiments, a suitable enzyme may contain about 10%, 15%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% bis-phosphorylated oligosaccharides per enzyme.

[0069] In some embodiments, recombinant I2S enzymes may be fused to a lysosomal targeting moiety that is capable of binding to a receptor on the surface of target cells. 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). The lysosomal targeting moiety may be conjugated or fused to an I2S protein or enzyme at the N-terminus, C-terminus or internally.

Production of Recombinant I2S Proteins

[0070] The present invention may be used to purify a recombinant I2S protein produced by various means. For example, an I2S protein may be recombinantly produced by utilizing a host cell system engineered to express an I2S-encoding nucleic acid. Alternatively, an I2S protein may be produced by activating an endogenous I2S gene.

[0071] It is contemplated that the present invention can be used to purify a recombinant I2S protein produced using various expression system. Suitable expression systems include, for example, egg, baculovirus, plant, yeast, or mammalian cells.

[0072] In some embodiments, I2S enzymes are produced in mammalian cells. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include 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 embryonic kidney line (HEK293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59,1977); human fibrosarcoma cell line (e.g., HT1080); baby hamster kidney cells (BHK21, 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; and a human hepatoma line (Hep G2).

[0073] In some embodiments, inventive methods according to the present invention are used to purify recombinant I2S enzymes produced from human cells (e.g., HT1080). In some embodiments, inventive methods according to the present invention are used to purify recombinant I2S enzymes produced from CHO cells.

[0074] Typically, cells that are engineered to express recombinant I2S may comprise a transgene that encodes a recombinant I2S protein described herein. It should be appreciated that the nucleic acids encoding recombinant I2S may contain regulatory sequences, gene control sequences, promoters, non-coding sequences and/or other appropriate sequences for expressing the recombinant I2S. Typically, the coding region is operably linked with one or more of these nucleic acid components.

[0075] "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, translation leader sequences, introns, and polyadenylation recognition sequences. Sometimes, "regulatory sequences" are also referred to as "gene control sequences."

[0076] "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.

[0077] 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.

[0078] 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.

[0079] Typically, the term "operatively linked" or "operably 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.

[0080] 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.

[0081] 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.

[0082] Once transfected or transduced into host cells, a suitable vector can express extrachromosomally (episomally) or integrate into the host cell's genome.

Activation of recombinant I2S proteins

[0083] Typically, a recombinant I2S enzyme is activated by the post-translational modification of a conserved cysteine (corresponding to amino acid 59 of mature human I2S) to formylglycine, also known as 2-amino-3-oxopropionic acid, or oxo-alanine. Such post-translational modification can be carried out by an enzyme known as Formylglycine Generating Enzyme (FGE). Thus, in some embodiments, recombinant I2S enzymes are produced in cells that also express FGE protein. In particular embodiments, recombinant I2S enzymes are produced in cells that have increased or enhanced expression of FGE protein. For example, cells may be engineered to over-express FGE in combination with recombinant I2S to facilitate the production of I2S preparations having high levels of active enzyme. In some embodiments, over-expression of FGE is achieved by expression (e.g., over-expression) of an exogenous FGE using standard recombinant technology. In some embodiments, over-expression of FGE is achieved by activated or enhanced expression of an endogenous FGE by, for example, activating or enhancing the promoter of the endogenous FGE gene. In some cases, the nucleic acid encoding recombinant I2S and the nucleic acid encoding a recombinant FGE protein are linked by a nucleic acid (*e.g.*, a spacer sequence) having a sequence corresponding to an internal ribosomal entry site.

[0084] Any FGE having ability to convert cysteine to formylglycine may be used in the present invention. Exemplary nucleic acid and amino acid sequences for FGE proteins are disclosed in US 2004-0229250, the entire contents relating to such sequences and the sequences themselves are incorporated herein by reference in their entireties. It should be appreciated that the nucleic acids encoding recombinant FGE may comprise regulatory sequences, gene control sequences, promoters, non-coding sequences and/or other appropriate sequences for expressing the FGE. Typically, the coding region is operably linked with one or more of these nucleic acid components.

Cell Culture Medium and Condition

[0085] Various cell culture medium and conditions may be used to produce a recombinant I2S protein. For example, a recombinant I2S protein may be produced in serum-containing or serum-free medium. In some embodiments, a recombinant I2S protein is produced in serum-free medium. In some embodiments, a recombinant I2S protein is produced in an animal free medium, i.e., a medium that lacks animal-derived components. In some embodiments, a recombinant I2S protein is produced in 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 such as serum, serum derived proteins (e.g., albumin or fetuin), and other 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.

[0086] 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).

[0087] Various cell culture conditions may be used to produce recombinant I2S proteins at large scale including, but not limited to, roller bottle cultures, bioreactor batch cultures and bioreactor fed-batch cultures. In some embodiments, recombinant I2S protein is produced by cells cultured in suspense. In some embodiments, recombinant I2S protein is produced by adherent cells.

[0088] Exemplary cell media and culture conditions are described in the Examples sections. Additional exemplary methods and compositions for producing recombinant I2S protein are described in the provisional application entitled "Methods and Compositions for Producing Recombinant Iduronate-2-Sulfatase" filed herewith on even date, the entire disclosure of which is hereby incorporated by reference.

Purification of Recombinant I2S Protein

[0089] In some embodiments, the present invention provides a method of purifying recombinant I2S protein from an impure preparation using a process based on one or more of anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography. In some embodiments, an inventive method according to the present invention involves less than 6 (e.g., less than 5, less than 4, or less than 3) chromatography steps. In some embodiments, an inventive method according to the present invention involves 2, 3, 4 or 5 chromatography steps. In some embodiments, an inventive method according to the present invention involves 4 chromatography steps. In some embodiments, an inventive method according to the present invention conducts anion-exchange chromatography, mixed-mode chromatography, cation-exchange chromatography, and hydrophobic interaction chromatography in that order.

Impure preparation

[0090] As used herein, an impure preparation can be any biological material including unprocessed biological material containing recombinant I2S protein. For example, an impure preparation may be unprocessed cell culture medium containing recombinant I2S protein secreted from the cells (e.g., mammalian cells) producing I2S protein or raw cell lysates containing I2S protein. In some embodiments, an impure preparation may be partially processed cell medium or cell lysates. For example, cell medium or cell lysates can be concentrated, diluted, treated with viral inactivation, viral processing or viral removal. In some embodiments, viral removal may utilize nanofiltration and/or chromatographic techniques, among others. In some embodiments, viral inactivation may utilize solvent inactivation, detergent inactivation, pasteurization, acidic pH inactivation, and/or ultraviolet inactivation, among others. Cell medium or cell lysates may also be treated with protease, DNases, and/or RNases to reduce the level of host cell protein and/or nucleic acids (e.g., DNA or RNA). In some embodiments, unprocessed or partially processed biological materials (e.g., cell medium or cell lysate) may be frozen and stored at a desired temperature (e.g., 2-8 °C, -4 °C, -25 °C, -75 °C) for a period time and then thawed for purification. As used herein, an impure preparation is also referred to as starting material or loading material.

Anion-exchange chromatography

[0091] In some embodiments, provided methods for purifying recombinant I2S include anion-exchange chromatography. In brief, anion exchange chromatography is a chromatographic technique which relies on charge–charge interactions between a negatively charged compound and a positively charged resin. In some embodiments, the anion-exchange chromatography is strong anion-exchange chromatography. In some embodiments, anion-exchange chromatography is employed as a first purification step for a therapeutic protein (e.g., recombinant I2S).

[0092] Exemplary anion exchange resins include, but are not limited to, quaternary amine resins or "Q–resins" (*e.g.*, Capto^{TM}-Q, Q–Sepharose$^{®}$, QAE Sephadex$^{®}$); diethylaminoethane (DEAE) resins (*e.g.*, DEAE–Trisacryl$^{®}$, DEAE Sepharose$^{®}$, benzoylated naphthoylated DEAE, diethylaminoethyl Sephacel$^{®}$); Amberjet$^{®}$ resins; Amberlyst$^{®}$ resins;

Amberlite® resins (*e.g.*, Amberlite® IRA–67, Amberlite® strongly basic, Amberlite® weakly basic), cholestyramine resin, ProPac® resins (*e.g.*, ProPac® SAX–10, ProPac® WAX–10, ProPac® WCX–10); TSK–GEL® resins (*e.g.*, TSKgel DEAE–NPR; TSKgel DEAE–5PW); and Acclaim® resins. In certain embodiments, the anion exchange resin is a Q resin.

[0093] Typical mobile phases for anionic exchange chromatography include relatively polar solutions, such as water, acetonitrile, organic alcohols such as methanol, ethanol, and isopropanol, or solutions containing 2-(N-morpholino)-ethanesulfonic acid (MES). Thus, in certain embodiments, the mobile phase includes about 0%, 1%, 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100% polar solution. In certain embodiments, the mobile phase comprises between about 1% to about 100%, about 5% to about 95%, about 10% to about 90%, about 20% to about 80%, about 30% to about 70%, or about 40% to about 60% polar solution at any given time during the course of the separation.

[0094] Generally, a mobile phase includes a salt. For example, a salt (e.g., sodium chloride) can elute a bound protein from an anion exchange column (e.g., the counter ion is chloride and it is exchanged for the target protein, which is then released). In some embodiments, the mobile phase includes a salt concentration between about 0 to about 1.0M, e.g., between about 0 to about 0.8M, between about 0 to about 0.6M, between about 0 to about 0.5M, between about 0 to about 0.4M, between about 0.05M to about 0.50M, between about 0.10M to about 0.45M, between about 0.10M to about 0.40M, or between about 0.15M to about 0.40M. In some embodiments, the mobile phase includes a salt concentration of approximately 0.01M, 0.02M, 0.03M, 0.04M, 0.05M, 0.06M, 0.07M, 0.08M, 0.09M, 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M, or 1.0M. In some embodiments, salt concentration in the mobile phase is a gradient (e.g., linear or non-linear gradient). In some embodiments, salt concentration in the mobile phase is constant. In some embodiments, salt concentration in the mobile phase may increase or decrease stepwise.

[0095] Typically, the mobile phase is buffered. In certain embodiments, the mobile phase is not buffered. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 14. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 10. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 7. In certain embodiments, the mobile phase is buffered to a pH of about

6.5. In certain embodiments, the mobile phase is buffered to a pH of about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10.

[0096] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to pH of about 5.0, 5.5, 6.0, 6.5, 7.0 or 7.5 and the conductivity of about 2 mS/cm, 4 mS/cm, 6 mS/cm, 8 mS/cm, 10 mS/cm, 12 mS/cm, 14 mS/cm, 16 mS/cm, 18 mS/cm, or 20 mS/cm prior to loading to the anion-exchange chromatography column (e.g., Q column). The pH may be adjusted using sodium acetate (e.g., 1M) and the conductivity may be adjusted using sodium chloride (e.g., 5M). Once loaded, an anion-exchange chromatography column may be washed using a wash buffer comprising salt (e.g., NaCl) concentration ranging from about 140 mM to about 200 mM (e.g., about 140 mM, 145 mM, 150 mM, 155 mM, 160 mM, 165 mM, 170 mM, 175 mM, 180 mM, 185 mM, 190 mM, 195 mM, or 200 mM) with pH of about 5.0-7.5 (e.g., about 5.0, 5.5, 6.0, 6.5, 7.0 or 7.5). An anion-exchange chromatography column may be eluted using an elution buffer comprising a linear NaCl gradient. A suitable exemplary linear NaCl gradient may contain a range from about 0-500 mM NaCl (e.g., about 0-400 mM, about 0-350 mM, about 0-300 mM, about 50-500 mM, about 150-500 mM, about 150-450 mM, about 150-400 mM).

Cation Exchange Chromatography

[0097] In some embodiments, provided methods for purifying recombinant I2S include cation-exchange chromatography. In brief, cation exchange chromatography is a chromatographic technique which relies on charge–charge interactions between a positively charged compound and a negatively charged resin. In some embodiments, the cation-exchange chromatography is strong cation-exchange chromatography.

[0098] Cation exchange chromatography is generally practiced with either a strong or weak cation exchange column, containing a sulfonium ion, or with a weak cation exchanger, having usually a carboxymethyl (CM) or carboxylate (CX) functional group. Many suitable cation exchange resins are known in the art and are commercially available and include, but are not limited to SP-Sepharose®, CM Sepharose®; Amberjet® resins; Amberlyst® resins; Amberlite® resins (*e.g.*, Amberlite® IRA120); ProPac® resins (*e.g.*, ProPac® SCX–10, ProPac® WCX–10, ProPac® WCX–10); TSK–GEL® resins (*e.g.*, TSKgel BioAssist S; TSKgel SP-2SW, TSKgel SP–5PW; TSKgel SP-NPR; TSKgel SCX; TSKgel SP-STAT;

TSKgel CM-5PW; TSKgel OApak-A; TSKgel CM-2SW, TSKgel CM-3SW, and TSKgel CM-STAT); and Acclaim® resins. In certain embodiments, the anion exchange resin is an SP-Sepharose resin®.

[0099] Typical mobile phases for cationic exchange chromatography include relatively polar solutions, such as water, acetonitrile, organic alcohols such as methanol, ethanol, and isopropanol, or solutions containing 2-(N-morpholino)-ethanesulfonic acid (MES). Thus, in certain embodiments, the mobile phase includes about 0%, 1%, 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100% polar solution. In certain embodiments, the mobile phase includes between about 1% to about 100%, about 5% to about 95%, about 10% to about 90%, about 20% to about 80%, about 30% to about 70%, or about 40% to about 60% polar solution at any given time during the course of the separation.

[0100] Generally, a mobile phase includes a salt. For example, a salt (e.g., sodium chloride, sodium phosphate, etc.) can elute a bound protein from an cation exchange column (e.g., the counter ion is sodium and it is exchanged for the target protein, which is then released). In some embodiments, the mobile phase includes a salt concentration between about 0 to about 1.0M, e.g., between about 0 to about 0.8M, between about 0 to about 0.6M, between about 0 to about 0.5M, between about 0 to about 0.4M, between about 0.05M to about 0.50M, between about 0.10M to about 0.45M, between about 0.10M to about 0.40M, or between about 0.15M to about 0.40M. In some embodiments, the mobile phase includes a salt concentration of approximately 0.01M, 0.02M, 0.03M, 0.04M, 0.05M, 0.06M, 0.07M, 0.08M, 0.09M, 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M, or 1.0M. In some embodiments, salt concentration in the mobile phase is a gradient (e.g., linear or non-linear gradient). In some embodiments, salt concentration in the mobile phase is constant. In some embodiments, salt concentration in the mobile phase may increase or decrease stepwise.

[0101] Typically, the mobile phase is buffered. In certain embodiments, the mobile phase is not buffered. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 14. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 10. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 7. In certain embodiments, the mobile phase is buffered to a pH of about 6.5. In certain embodiments, the mobile phase is buffered to a pH of about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10.

[0102] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to conductivity ranging between about 1 mS/cm and 20 mS/cm (e.g., between about 1 mS/cm and 15 mS/cm, between about 1 mS/cm and 10 mS/cm, between about 1 mS/cm and 8 mS/cm, between about 1 mS/cm and 6 mS/cm, between about 1 mS/cm and 4 mS/cm, between about 2 mS/cm and 4 mS/cm) prior to loading to the cation-exchange chromatography column (e.g., SP column). In particular embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to conductivity ranging between n about 2 mS/cm and 4 mS/cm (e.g., 2, 2.5, 3, 3.5, or 4 mS/cm) prior to loading to the cation-exchange chromatography column (e.g., SP column). The conductivity may be adjusted by diluting an impure preparation or an intermediate eluate or flow-through with H_2O at, e.g., 1:1, 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 2.0:1, 2.5:1, 3.0:1, 4.0:1, 5.0:1, or 10:1 ratio. The conductivity may also be adjusted by dialfiltration into a desired buffer. In some embodiments, a cation-exchange chromatography column is run at a pH of about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0 or 6.5). In some embodiments, a cation-exchange chromatography column is run with a buffer comprising phosphate (e.g., NaPO4) concentration ranging from about 0.01 M to about 0.1 M (e.g., about 0.01 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M, 0.06 M, 0.07 M, 0.08 M, 0.09 M, or 0.1 M). In some embodiments, a suitable pH is about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0, or 6.5).

Mixed Mode Chromatography

[0103] Hydroxyapatite chromatography (HA) is considered to be a “pseudo-affinity” chromatography or “mixed-mode” ion exchange and may be used in accordance with the present invention. Hydroxyapatite is a unique form of calcium phosphate used in fractionation and purification of biological molecules. In some cases, crystalline hydroxyapatite may be used, although the fragility of the crystals may limit flow rates and/or column longevity. Two types of chemically pure ceramic hydroxyapatite, CHT ceramic hydroxyapatite Types I and II are macroporous, spherical and can be used at high flow rates and pressures. Type I generally has a high protein binding capacity, while Type II generally has a lower binding capacity for proteins. In general, the formula of hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$ (Kawasaki, et al 1985). The functional groups include positively charged pairs of crystal calcium ions (C-sites) and clusters of six negatively charged oxygen atoms associated with triplets of crystal phosphates (P-sites). C-sites, P-sites and hydroxyls are distributed in a fixed pattern on the crystal surface, generally leading to complex interactions with proteins and other molecules.

[0104] A sample may be loaded onto an HA column in low ionic strength phosphate buffer (e.g., 1-10 mM sodium or potassium phosphate) at or near neutral pH. In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to phosphate (e.g., NaPO4) concentration ranging from about 0.001 M to about 0.01 M (e.g., about 0.001 M, 0.002 M, 0.003 M, 0.004 M, 0.005 M, 0.006 M, 0.007 M, 0.008 M, 0.009 M, or 0.01 M) and pH of about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0, or 6.5) prior to loading the mixed-mode chromatography column (e.g., HA column). The loaded HA column are typically washed with a wash buffer having a phosphate concentration comparable to that of the loading buffer. In some embodiments, the mixed-mode chromatography column (e.g., HA column), once loaded, is washed with a wash buffer containing phosphate (e.g., 1-10 mM sodium or potassium phosphate) at or near neutral pH. For example, a suitable wash buffer may have a phosphate concentration of about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, or 10 mM. In some embodiments, it may be desirable to increase the amount of phosphate in the wash buffer to create a more stringent wash condition. It is contemplated that the M6P levels, in particular di-M6P levels, on the surface of I2S proteins are important for lysosomal targeting. Increased phosphate concentration in the wash buffer may selectively retain I2S proteins with high levels of M6P, in particular, di-M6P on the HA column. Thus, in some embodiments, a desired wash buffer may have a phosphate concentration of or greater than 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM. In some embodiments, the loaded mixed-mode chromatography column (e.g., HA column) is washed with a wash buffer having a phosphate concentration ranging from about 10-20 mM (e.g., about 10-18 mM, 10-16 mM, 10-15 mM, 12-20 mM, 14-18 mM, 14-16 mM). In some embodiments, the loaded mixed-mode chromatography column (e.g., HA column) is washed with a wash buffer having a phosphate concentration of or greater than 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM.

[0105] Elution from an HA column is typically achieved with a gradient phosphate buffer. For example, a suitable elution buffer may have a phosphate gradient of approximately 1-400 mM (e.g., 1-300 mM, 1-200 mM, 1-150 mM, 1-100 mM, 10-350 mM, 10-300 mM, 10-250 mM, 10-200 mM, 10-150 mM, 10-140 mM, 10-130 mM, 10-120 mM, 10-110 mM, 10-100 mM, 10-90 mM, 10-80 mM, 10-70 mM, 10-60 mM, 10-50 mM) sodium phosphate. In some embodiments, elution from an HA column is achieved by stepwise increasing the phosphate concentration in the elution buffer. In some embodiments, stepwise elution buffers may have a phosphate concentration selected from 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM. In some embodiments, elution from a mixed-mode chromatography column (e.g., HA column) is achieved by an elution buffer having a phosphate (e.g., sodium phosphate) concentration ranging from about 50 mM to about 150 mM (e.g., selected from a phosphate (e.g., sodium phosphate) concentration of about 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 110 mM, 120 mM, 130 mM, 140 mM, 150 mM, and combination thereof).

[0106] It will be appreciated that many different combinations of conditions for HA chromatography are known and may be used to adjust the parameters to be suitable for a particular protein of interest (e.g., recombinant I2S).

Hydrophobic Interaction Chromatography

[0107] Hydrophobic Interaction Chromatography (HIC) is a separation technique that uses the properties of hydrophobicity to separate proteins from one another. In this type of chromatography, hydrophobic groups such as phenyl, octyl, or butyl, are attached to the stationary column. Proteins that pass through the column that have hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column. HIC columns are known, and include for example, Phenyl Sepharose

[0108] HIC separations are often designed using the opposite conditions of those used in ion exchange chromatography. In general, a buffer with a high ionic strength, usually ammonium sulfate, is initially applied to the column. The salt in the buffer reduces the solvation of sample solutes thus as solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium. The stationary phase is generally designed to form hydrophobic interactions with other molecules. These interactions are generally too weak in water, however, addition of salts (e.g., Na_2SO_4, K_2SO_4, $(NH_4)_2SO_4$, NaCl, NH_4Cl, NaBr, and NaSCN) to the buffer results in hydrophobic interactions. In some embodiments, the mobile phase includes a salt concentration between about 0.1M to about 3.0M, e.g., between about

0.1M to about 1.5M, between about 0.2M to about 0.8M, or between about 0.3M to about 0.5M.

[0109] In certain embodiments, the mobile phase is buffered. In certain embodiments, the mobile phase is not buffered. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 14. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 10. In certain embodiments, the mobile phase is buffered to a pH between about 5 to about 7. In certain embodiments, the mobile phase is buffered to a pH of about 5.0.

[0110] In some embodiments, an impure preparation or an intermediate eluate or flow-through is adjusted to salt (e.g., NaCl) concentration ranging from about 0.5 M to about 2.0 M (e.g., about 0.5 M, 1.0 M, 1.1 M, 1.2 M, 1.3 M, 1.4 M, 1.5 M, 1.6 M, 1.7 M, 1.8 M, 1.9 M, or 2.0 M) at pH of about 4.5-6.0 (e.g., about 4.5, 5.0, 5.5, or 6.0) prior to loading onto the hydrophobic interaction chromatography column (e.g., phenyl column). Once loaded, a hydrophobic interaction chromatography column may be washed using a wash buffer comprising salt (e.g., NaCl) concentration ranging from about 0.5 M to about 2.0 M (e.g., about 0.5 M, 1.0 M, 1.1 M, 1.2 M, 1.3 M, 1.4 M, 1.5 M, 1.6 M, 1.7 M, 1.8 M, 1.9 M, or 2.0 M) at pH of about 4.5-6.0 (e.g., about 4.5, 5.0, 5.5, or 6.0). In some embodiments, the hydrophobic interaction chromatography column is eluted using a elution buffer comprising salt (e.g., NaCl) concentration ranging from about 0.1 M to about 0.5 M (e.g,. about 0.1 M, 0.2 M, 0.3 M, 0.4 M, or 0.5 M) at pH of about 4.5-6.0 (e.g., about 4.5, 5.0, 5.5, or 6.0).

Characterization of Purified I2S Proteins

[0111] Purified recombinant I2S protein may be characterized using various methods.

Purity

[0112] The purity of purified recombinant I2S protein is typically measure by the level of various impurities (e.g., host cell protein or host cell DNA) present in the final product. For example, the level of host cell protein (HCP) may be measured by ELISA or SDS-PAGE. In some embodiments, the purified recombinant I2S protein contains less than 150 ng HCP/mg I2S protein (e.g., less than 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 30, 20, 10 ng HCP/mg I2S protein). In some embodiments, the purified recombinant I2S protein, when subject to SDS-PAGE with silver staining, has no new bands with intensity greater than the 0.05%, 0.01%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, or 0.5% assay control. Various assay controls may be used, in particular, those acceptable to regulatory agencies such as FDA.

Specific Activity

[0113] Purified recombinant I2S protein may also be characterized by evaluating functional and/or biological activity. The enzyme activity of a recombinant I2S composition may be determined using methods known in the art. Typically the methods involve detecting the removal of sulfate from a synthetic substrate, which is known as sulphate release assay. One example of an enzyme activity assay involves the use of ion chromatography. This method quantifies the amount of sulfate ions that are enzymatically released by recombinant I2S from a substrate. The substrate may be a natural substrate or a synthetic substrate. In some cases, the substrate is heparin sulfate (e.g., heparin disaccharide), dermatan sulfate, or a functional equivalent thereof. Typically, the released sulfate ion is analyzed by ion chromatography with a conductivity detector. In this example, the results may be expressed as U/mg of protein where 1 Unit is defined as the quantity of enzyme required to release 1 μmole sulfate ion per hour from the substrate. In some embodiments, purified recombinant I2S protein has a specific activity, as measured by *in vitro* sulfate release activity assay using heparin disaccharide as substrate, ranging from about 0-100 U/mg, about 10-100 U/mg, about 10-80 U/mg, about 20-80 U/mg, about 20-70 U/mg, about 20-60 U/mg, about 20-50 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 40-100 U/mg, about 40-90 U/mg, about 40-80 U/mg, about 40-70 U/mg, about 40-60 U/mg. In some embodiments, purified recombinant I2S protein has a specific activity, as measured by *in vitro* sulfate release activity assay using heparin disaccharide as substrate, of at least about 5 U/mg, about 10 U/mg, about 15 U/mg, about 20 U/mg, about 25 U/mg, about 30 U/mg, about 35 U/mg, about 40 U/mg, about 45 U/mg, about 50 U/mg, about 55 U/mg, about 60 U/mg, about 65 U/mg, about 70 U/mg, about 75 U/mg, about 80 U/mg, about 85 U/mg, about 90 U/mg, about 95 U/mg, or about 100 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 disaccharide. 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.

[0114] 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 0.5 U/mg, 1.0 U/mg, 1.5 U/mg, 2 U/mg, 2.5 U/mg, 3 U/mg, 4 U/mg, 5 U/mg, 6 U/mg, 7 U/mg, 8 U/mg, 9 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. Specific activity may then calculated by dividing the enzyme activity by the protein concentration.

[0115] In either example, the protein concentration of a recombinant I2S composition may be determined by any suitable method known in the art for determining protein concentrations. In some cases, the protein concentration is determined by an ultraviolet light absorbance assay. Such absorbance assays are typically conducted at about a 280 nm wavelength (A_{280}).

Charge Profile

[0116] Purified recombinant I2S may be characterized by the charge profile associated with the protein. Typically, protein charge profile reflects the pattern of residue side chain charges, typically present on the surface of the protein. Charge profile may be determined by performing an ion exchange (IEX) chromatography (e.g., HPLC) assay on the protein. In some embodiments, a "charge profile" refers to a set of values representing the amount of protein that elutes from an ion exchange column at a point in time after addition to the column of a mobile phase containing an exchange ion.

[0117] Typically, a suitable ion exchange column is an anion exchange column. For example, a charge profile may be determined by strong anion exchange (SAX) chromatography using a high performance liquid chromatography (HPLC) system. In general, recombinant I2S adsorbs onto the fixed positive charge of a strong anion exchange column and a gradient of increasing ionic strength using a mobile phase at a predetermined flow rate elutes recombinant I2S species from the column in proportion to the strength of their ionic interaction with the positively charged column. More negatively charged (more acidic) I2S species elute later than less negatively charged (less acid) I2S species. The concentration of proteins in the eluate are detected by ultraviolet light absorbance (at 280 nm).

[0118] In some embodiments, recombinant I2S adsorbs at about pH 8.0 in 20 mM TRIS-HCl onto the fixed positive charge of a Mini Q PE column and a gradient of increasing ionic strength using a mobile phase consisting of 20 mM TRIS-HCL, 1 M sodium chloride, pH 8.0 at a flow rate of 0.8 ml/min elutes recombinant I2S species from the column in proportion to the strength of their ionic interaction with the positively charged column.

[0119] In some embodiments, a charge profile may be depicted by a chromatogram of absorbance units versus time after elution from the HPLC column. The chromatogram may comprise a set of one or more peaks, with each peak in the set identifying a subpopulation of recombinant I2Ss of the composition that have similar surface charges.

[0120] In some embodiments, a purified I2S protein composition exhibits at least six peaks in its charge profile. An exemplary charge profile of I2S is depicted in the Examples section and in Figure 11. As shown in Figure 11, six peaks are labeled (A to F) in the order of increasing negative charge and decreasing contribution to total peak area of the chromatogram. In some embodiments, the charge profile for a purified recombinant I2S composition contains a different number, size, shape or time interval of peaks depending on the amount of negative or positive charges on the surface of the protein. In some embodiments, a recombinant I2S composition has a charge profile that has fewer than 6 (e.g., fewer than 5, 4, 3, or 2) peaks. In some embodiments, a charge profile of recombinant I2S may have 5, 4, 3, 2, or 1 peak(s). For example, any one, two, three, four, or five of peaks A, B, C, D, E, and F may be absent or reduced in a purified recombinant I2S protein composition. Typically, a charge profile is considered more homogenous if there are fewer peaks.

Glycan Mapping

[0121] In some embodiments, a purified recombinant I2S protein may be characterized by their proteoglycan composition, typically referred to as glycan mapping. Without wishing to be bound by any theory, it is thought that glycan linkage along with the shape and complexity of the branch structure may impact *in vivo* clearance, lysosomal targeting, bioavailability, and/or efficacy.

[0122] Typically, a glycan map may be determined by enzymatic digestion and subsequent chromatographic analysis. Various enzymes may be used for enzymatic digestion including, but not limited to, suitable glycosylases, peptidases (e.g., Endopeptidases, Exopeptidases), proteases, and phosphatases. In some embodiments, a suitable enzyme is alkaline phosphatase. In some embodiments, a suitable enzyme is neuraminidase. Glycans (e.g., phosphoglycans) may be detected by chromatographic analysis. For example, phosphoglycans may be detected by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD) or size exclusion High Performance Liquid Chromatography (HPLC). The quantity of glycan (e.g., phosphoglycan) represented by each peak on a glycan map may be calculated using a standard curve of glycan (e.g., phosphoglycan), according to methods known in the art and disclosed herein.

[0123] In some embodiments, a purified I2S according to the present invention exhibits a glycan map comprising seven peak groups indicative of neutral (peak group 1), mono-sialylated (peak group 2), di-sialylated (peak group 3), monophosphorylated (peak group 4), tri-sialylated (peak group 5), tetra-sialylated (peak group 6), and diphosphorylated (peak group 7) I2S protein, respectively. Exemplary glycan maps of I2S are depicted in Figure 10. In some embodiments, a purified recombinant I2S has a glycan map that has fewer than 7 peak groups (e.g., a glycan map with 6, 5, 4, 3, or 2 peaks groups). In some embodiments, a purified recombinant I2S has a glycan map that has more than 7 peak groups (e.g., 8, 9, 10, 11, 12 or more).

[0124] The relative amount of glycan corresponding to each peak group may be determined based on the peak group area relative to the corresponding peak group area in a predetermined reference standard. In some embodiments, peak group 1 (neutral) may have the peak group area ranging from about 40-120% (e.g., about 40-115%, about 40-110%, about 40-100%, about 45-120%, about 45-115%, about 45-110%, about 45-105%, about 45-100%, about 50-120%, about 50-110%) relative to the corresponding peak group area in a reference standard. In some embodiments, peak group 2 (monosialylated) may have the peak group area ranging from about 80-140% (e.g., about 80-135%, about 80-130%, about 80-125%, about 90-140%, about 90-135%, about 90-130%, about 90-120%, about 100-140%) relative to the corresponding peak group area in the reference standard. In some embodiments, peak group 3 (disialylated) may have the peak group area ranging from about 80-110% (e.g., about 80-105%, about 80-100%, about 85-105%, about 85-100%) relative to the corresponding peak group area in the reference standard. In some embodiments, peak group 4 (monophosphorylated) may have the peak group area ranging from about 100-550% (e.g., about 100-525%, about 100-500%, about 100-450%, about 150-550%, about 150-500%, about 150-450%, about 200-550%, about 200-500%, about 200-450%, about 250-550%, about 250-500%, about 250-450%, or about 250-400%) relative to the corresponding peak group area in the reference standard. In some embodiments, peak group 5 (tri-sialylated) may have the peak group area ranging from about 70-110% (e.g., about 70-105%, about 70-100%, about 70-95%, about 70-90%, about 80-110%, about 80-105%, about 80-100%, or about 80-95%) relative to the corresponding peak group area in the reference standard. In some embodiments, peak group 6 (tetra-sialylated) may have the peak group area ranging from about 90-130% (e.g., about 90-125%, about 90-120%, about 90-115%, about 90-110%, about 100-130%, about 100-125%, or about 100-120%) relative to the corresponding peak group area in the reference standard. In some embodiments, peak group 7 (diphosphorylated) may have with the peak group area ranging from about 70-130% (e.g., about 70-125%, about 70-120%, about 70-115%, about 70-110%, about 80-130%, about 80-125%, about 80-120%, about 80-115%, about 80-110%, about 90-130%, about 90-125%, about 90-120%, about 90-115%, about 90-110%) relative to the corresponding peak group area in the reference standard. Various reference standards for glycan mapping are known in the art and can be used to practice the present invention. Typically, peak group 7 (diphosphorylated) corresponds to the level of di-M6P on the surface of the purified recombinant I2S protein.

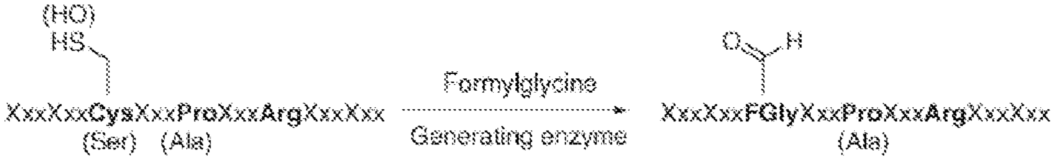
[0125] It is contemplated that the glycosylation pattern of a purified I2S impacts the lysosomal targeting. Various *in vitro* cellular uptake assays are known in the art and can be used to practice the present invention. For example, to evaluate the uptake of I2S by M6P receptors, cellular uptake assays are performed using human fibroblasts expressing M6P receptors on their surface. The internalized amount of I2S can be measured by a ELISA method. In some embodiments, a purified recombinant I2S protein according to the present invention is characterized with cellular uptake of greater than 70%, 75%, 80%, 85%, 90%, 95%, as determined by an *in vitro* uptake assay.

Peptide Mapping

[0126] In some embodiments, peptide mapping may be used to characterize amino acid composition, post-translational modifications, and/or cellular processing; such as cleavage of a signal peptide, formylglycine conversion and/or glycosylation. Typically, a recombinant protein may be broken into discrete peptide fragments, either through controlled or random breakage, to produce a pattern or peptide map. In some cases, a purified I2S protein may be first subjected to enzymatic digest prior to analytic analysis. Digestion may be performed using a peptidase, glycoside hydrolase, phosphatase, lipase or protease and/or combinations thereof, prior to analytic analysis. The structural composition of peptides may be determined using methods well known in the art. Exemplary methods include, but are not limited to, Mass spectrometry, Nuclear Magnetic Resonance (NMR) or HPLC.

Percent Formylglycine Conversion

[0127] Peptide mapping can be used to determine Percent FGly conversion. As discussed above, I2S activation requires Cysteine (corresponding to position 59 of the 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:

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

[0128] To calculate %FG, a recombinant I2S protein may be digested into short peptides using a protease (e.g., trypsin or chymotrypsin). Short peptides may be separated and characterized using, e.g., size exclusion High Performance Liquid Chromatography (HPLC). The peptide containing the position corresponding to position 59 of the mature human I2S may be 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 can be calculated.

[0129] In some embodiments, a purified recombinant I2S protein according to the present invention 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, a purified recombinant I2S protein according to the present invention has substantially 100% conversion of the cysteine residue corresponding to Cys59 of human I2S (SEQ ID NO:1) to C_α-formylglycine (FGly).

Sialic Acid Content

[0130] In some embodiments, a purified recombinant I2S protein may be characterized by their sialic acid composition. Without wishing to be bound by theory, it is contemplated that sialic acid residues on proteins may prevent, reduce or inhibit their rapid *in vivo* clearance via the asialoglycoprotein receptors that are present on hepatocytes. Thus, it is thought that recombinant proteins that have relatively high sialic acid content typically have a relatively long circulation time *in vivo*.

[0131] In some embodiments, the sialic acid content of a purified recombinant I2S protein may be determined using methods well known in the art. For example, the sialic acid content of a recombinant I2S protein may be determined by enzymatic digestion and subsequent chromatographic analysis. Enzymatic digestion may be accomplished using any suitable sialidase. In some cases, the digestion is performed by a glycoside hydrolase enzyme, such as neuraminidase. Sialic acid may be detected by chromatographic analysis such as, for example, High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD). The quantity of sialic acid in a recombinant I2S composition may be calculated using a standard curve of sialic acid, according to methods known in the art and disclosed herein.

[0132] In some embodiments, the sialic acid content of a purified recombinant I2S protein may be greater than 16 mol/mol. The units "mol/mol" in the context of sialic acid content refers to moles of sialic acid residue per mole of enzyme. In some cases, the sialic acid content of a recombinant I2S protein is or greater than about 16.5 mol/mol, about 17 mol/mol, about 18 mol/mol, about 19 mol/mol, about 20 mol/mol, about 21 mol/mol, about 22 mol/mol or more. In some embodiments, the sialic acid content of a purified recombinant I2S protein may be in a range between about 16-20 mol/mol, 16-21 mol/mol, about 16-22 mol/mol, 16-23 mol/mol, 16-24 mol/mol, about 16-25 mol/mol, about 17-20 mol/mol, 17-21 mol/mol, about 17-22 mol/mol, 17-23 mol/mol, 17-24 mol/mol, or about 17-25 mol/mol.

Pharmaceutical Composition and Administration

[0133] 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)).

[0134] In some embodiments, a recombinant I2S or a pharmaceutical composition containing the same is administered to a subject by intravenous administration.

[0135] 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.

[0136] 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^{TM} and Genject^{TM} devices); injector pens (such as the GenPen^{TM}); needleless devices (e.g., MediJector^{TM} and BioJector^{TM}); and subcutaneous patch delivery systems.

[0137] 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)).

[0138] 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).

[0139] 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.

[0140] 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.

[0141] 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.*

[0142] 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.

[0143] 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.

[0144] 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.

[0145] 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.

[0146] 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: Recombinant I2S AF Capture and Purification Process

[0147] This example demonstrates a simplified downstream purification process may be used to capture and purify recombinant I2S produced in serum-free medium. An exemplary purification scheme is depicted in Figure 1.

[0148] A cell line stably expressing an iduronate-2-sulfatase enzyme (I2S) and formylglycine generating enzyme (FGE) was developed. Generation and characterization of exemplary cell lines are described in the U.S. Provisional Application entitled "Cells for Producing Recombinant Iduronate-2-Sulfatase" filed on even date herewith, the entire contents of which is hereby incorporated by reference. Briefly, 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:5.

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

SEQ ID NO: 5
Full-length human FGE precursor:
MAAPALGLVCGRCPELGLVLLLLLLSLLCGAAGSQEAGTGAGAGSLAGSCGCGTPQ
RPGAHGSSAAAHRYSREANAPGPVPGERQLAHSKMVPIPAGVFTMGTDDPQIKQDG
EAPARRVTIDAFYMDAYEVSNTEFEKFVNSTGYLTEAEKFGDSFVFEGMLSEQVKTN
IQQAVAAAPWWLPVKGANWRHPEGPDSTILHRPDHPVLHVSWNDAVAYCTWAGK
RLPTEAEWEYSCRGGLHNRLFPWGNKLQPKGQHYANIWQGEFPVTNTGEDGFQGT
APVDAFPPNGYGLYNIVGNAWEWTSDWWTVHHSVEETLNPKGPPSGKDRVKKGGS
YMCHRSYCYRYRCAARSQNTPDSSASNLGFRCAADRLPTMD

[0149] After synthesis of the full length I2S enzyme, the 25 amino acid signal peptide is removed and a soluble mature I2S enzyme is secreted from the cell.

[0150] A chemically defined media (serum free/animal-component free; AF) was used in the bioreactor process.

[0151] Individual harvest material was reduced in volume and buffer exchanged through an ultrafiltration/dialfiltration process. The material, termed unpurified bulk (UPB), was frozen at -50°C per individual harvest. The downstream purification process began with the thaw and pool of unpurified bulk and included successive viral inactivation, anion exchange (Capto Q), mixed mode (ceramic hydroxyapatite), cation exchange (SP Sepharose) and hydrophobic interaction (Phenyl Sepharose) chromatography steps followed by viral filtration, and final concentration and diafiltration step. In particular, this purification process utilized Q, hydroxyapatite, SP and Phenyl chromatographic modalities. Protein G Chromatography and Size Exclusion Chromatography traditionally used in I2S purification process were removed. Exemplary steps are shown in Table 3.

Table 3: Exemplary Steps of Purification Process

Harvest
UF/DF
Frozen Storage
Thaw, Pool and Depth Filtration ↓ Solvent/Detergent Viral Inactivation ↓ Anion-exchange Chromatography ↓ Mixed-mode Chromatography ↓ ------------------- ↓ Dilution (1-1.2 fold) ↓ Cation-exchange Chromatography ↓ Hydrophobic interaction Chromatography

↓

↓

↓

Viral Removal Filtration

↓

UF/DF

↓

Drug Substance

[0152] Purified I2S protein were assessed for purity by peptide mapping, SDS-PAGE (Silver), size exclusion HPLC. Enzyme specific activity, formylglycine content, sialic acid content, glycan map, charge profiles were determined using standard methods. Exemplary results are shown in Table 4.

Table 4: Analysis of Purified Recombinant I2S Protein

Assay		**Purified I2S (10 L scale) Min-Max (n)**
Peptide Mapping	L1	100-105% (n=3)
	L10	98-100% (n=3)
	L12	102-102% (n=3)
	L13	96-97% (n=3)
	L14	102-103% (n=3)
	L17	101-101% (n=3)
	L20	102-103% (n=3)
Host Cell Protein		≤62.5 (n=5)
SDS-PAGE (Silver)		Conforms
Ion Exchange HPLC	%Peak A	69-69% (n=2)
	%Peak B	20-21% (n=2)
	%Peak E+F	10-11% (n=2)
Size Exclusion HPLC		99.9-99.9% (n=5)
Cellular Uptake (Bioassay)		85, 95% and 97% (n=3)
% Formylglycine		87-95% (n=5)
Specific Activity		62-78 (n=5)
Glycan Map	Pk Grp 3	88-93% (n=5)

	Pk Grp 5	72-110% (n=5)
	Pk Grp 6	124-133% (n=5)
	Pk Grp 7	78-87% (n=5)
	Total Area	94-116% (n=5)
Sialic Acid		16-22 (n=4)
Endotoxin		<0.04-<0.05 (n=2)
Bioburden		0.00-0.00 (n=2)

[0153] An exemplary peptide map as compared to commercially available I2S reference is shown in Figure 2. Exemplary SDS-PAGE (Silver) analysis results are shown in Figure 3. Typically, using a process described herein, the HCP concentration of drug substance (DS) was <100 ppm, meeting the <l00 ppm specification required in many markets including the US. The SEC of DS was ≥99.5%, also meeting the current >99.3% marketing specification requirement in many markets. Exemplary charge profile is shown in Figure 4. Exemplary glycan map is shown in Figure 5. In particular, the glycan map of purified I2S includes seven peak groups, eluting according to an increasing amount of negative charges derived from sialic acid and mannose-6-phosphate residues, representing in the order of elution, neutrals, mono-, disialylated, monophosphorylated, trisialylated and hybrid (monosialylated and capped M6P), tetrasialylated and hybrid (disialylated and capped M6P) and diphosphorylated glycans.

[0154] Taken together, this example demonstrates that a simplified four-column purification process can be used to successfully purify recombinant I2S produced in animal free medium at large scale.

Example 2: Harvest and Viral Inactivation Stability Studies of Recombinant I2S AF

[0155] The objective of this study was to evaluate the effects of temperature hold time and freeze-thaw cycles on the stability of recombinant I2S clarified harvest.

[0156] Clarified harvest samples were stored at ambient and 2-8°C for up to seven days and the viral inactivated UPB samples were held at ambient for up to 24 hours. Freeze-thaw samples on clarified harvests were frozen at -20°C, -50°C, and -80°C and experienced freeze-thaw for up to three cycles. Stability was gauged using Western blot, SEC HPLC, and activity assay.

[0157] I2S-AF harvest material was produced from the 2D cell line by CCPD using a B. Braun 20L bioreactor with a centrifuge retention device and a desired bleeding rate. For the temperature holding study, each clarified harvest was stored at ambient and 2-8°C and sampled at selected hold times. Sampling amounts and hold times are listed in Table 5. Freeze-thaw samples were stored at -20°C, -50°C, and -80°C and thawed using a water bath at 25°C.

Table 5. Clarified Harvest Hold Point Stability

	Samples	Holding Temperature	Holding Time (Days)
Clarified Harvest 12	15 x 0.5 mL	2-8 °C	T=0, 24h, 76h, 120h, 168h
	15 x 0.5 mL	Ambient	T=0, 24h, 76h, 120h, 168h
	9 x 0.5 mL	-20 °C, -50 °C, and -80 °C	Freeze/Thaw 1, 2, and 3
Clarified Harvest 18	15 x 0.5 mL	2-8 °C	T=0, 24h, 76h, 120h, 168h
	15 x 0.5 mL	Ambient	T=0, 24h, 76h, 120h, 168h
	9 x 0.5 mL	-20 °C, -50 °C, and -80 °C	Freeze/Thaw 1, 2, and 3

[0158] The viral inactivation step occurred at the unpurified bulk step prior to loading the first column. UPB was produced by concentrating and buffer exchange of clarified harvest. UF/DF was performed using a Pall 1 sq. ft. Centramate system and buffer exchanged into 10 mM MES, 155 mM NaCl, pH=6.5. The viral inactivation step added 1% Tween 80 and 0.3% TnBP, filtered using Durapore syringe filters for each time point. Samples were taken at each time point listed in Table 6 and frozen at -80°C. Samples from the clarified harvest hold point and freeze-thaw studies were tested by Western blot and activity (4-MU assay). UPB samples from the viral inactivation were tested for purity by SEC HPLC. The hold point activity results from Harvest 12 and 18 on Table 5 showed no significant changes up to 7 days of storage at ambient and 2-8°C for both harvests. There were no significant changes seen in Harvest 12 activity for up to 3 freeze-thaw cycles stored at -20°C, -50°C, and -80°C.

Table 6. Viral Inactivation of Unpurified Bulk

	Samples	Holding Temperature	Holding Time (Days)
Viral Inactivation	9 x 0.5 mL	Ambient, Control	T=0, 6h, 24h
	9 x 0.5 mL	Ambient, Viral Inactivation	T=0, 6h, 24h

[0159]

[0160] Activity and SEC-HPLC for the stability of the viral inactivation UPB step are described in Figures 6 and 7. This shows that there were no issues in viral inactivation stability based on activity and purity for up to 24 hours.

[0161] In summary, based on the stability analysis described herein, clarified harvest can be stored at 2-8°C (for example, for up to 7 days) without significant changes in harvest quality. Clarified harvests can experience multiple freeze-thaw cycles and stored at -20°C, -50°C, and -80°C temperatures with no significant changes in stability. Based on SEC HPLC purity results, viral inactivation at the UPB step can occur at ambient temperature (e.g., for up to 24 hours) with no changes in activity and purity.

Example 3: Purification and Analysis of Animal-Free IL CD Media Confirmation Run

[0162] The objective of this study was to perform purification from pooled harvest of I2S-AF produced in an animal-free perfusion using chemically defined media and to characterize the drug substance.

[0163] This study evaluated I2S-AF purification process performance and drug substance (DS) produced from a chemically defined medium bioreactor.

Cell Culture

[0164] The I2S-AF material was produced from cell line 2D expressing I2S and formylglycine generating enzyme (FGE)) as described in Example 1. The material was produced in CCPD in a 1L Das Gip spin filter bioreactor using a chemically defined serum free media. Individual bags from each clarified harvest (HI-21) were received frozen at -20°C and thawed at 2-8°C overnight. Equal volumes of each clarified harvest was pooled to represent an entire harvest pool, then 0.2μm filtered and concentrated using 30 kD Pall Omega Centramate cassette with a total membrane area of 1 ft^2. The unpurified bulk (UPB) was 0.2 um filtered and frozen prior to use.

Purification

[0165] Exemplary column specifications and loading are described in Table 7. The Q Sepharose was loaded at a target of 3 g/L by titer. Subsequent columns were loaded at 100% from the previous column elution and no material removed.

Table 7. Column and Loading Specifications

Column	Column Dimensions (cm x cm)	Column Volume (mL)	Column Load (g/L resin by I2S)	Column Load (mg)
Q Sepharose	2.6 x 25	133	3	399
HA Type II, 80 µm	1.6 x 30	60	5.5	330
Phenyl Sepharose	1.6 x 23	46	5.6	258

[0166] One purification run was performed using UPB from pooling harvests 1 through 21 from the bioreactor. UPB was thawed at 2-8°C overnight and pooled by equal volume from each harvest.

[0167] Individual column process steps and buffer formulations can be found in Tables 8-11. The pooled UPB was filtered using a 0.2 um bottle filter system, adjusted to pH 6.5 using 1 M sodium acetate, and conductivity adjusted to 16 mS/cm with 5 M sodium chloride prior to loading onto the Q Sepharose FF column. The Q Sepharose elution was adjusted to 0.001 M $NaP0_4$ using 0.25M $NaP0_4$, pH 5.5 and filtered with a 0.22 um PES bottle top filter prior to loading onto the HA column. The HA elution conductivity was adjusted to 1.55 M NaCl with 5 M NaCl and pH adjusted to pH 5.5 with 1 M sodium acetate. The adjustment time was approximately 1 hour. The adjusted pool was filtered using a 0.22 um PES bottle top filter prior to loading onto the Phenyl Sepharose column. The Phenyl elution was concentrated 4X and diafiltered 6X into 0.02 M $NaP0_4$, 0.137 M NaCl, pH 6.0. The diafiltered product was adjusted to 2.0 g/L and formulated with 0.2% Polysorbate 20 to generate mock drug substance. A mock pool of H1-20 of the DS was created for additional characterization.

Table 8. Exemplary Process Details for Q Sepharose FF Chromatography

Process Step	Flow rate (cm/hr)	CV	Buffers
Sanitization	150	3	0.5 N NaOH
Equilibration	150	4	0.01 M MES, 0.155 M NaCl, ph 6.5
Wash 1	150	2	0.01 M MES, 0.155 M NaCl, ph 6.5
Wash 2	150	3	0.01 M MES, 0.155 M NaCl, ph 5.5
Elution	150	3	0.01 M MES, 0.50 M NaCl, ph 5.5
Clean/Strip	150	4	1.0 M NaOH, 2 M NaCl
Store	150	4	0.0 N NaOH

Table 9. Exemplary Process Details for HA Chromatography

Process Step	Flow rate (cm/hr)	CV	Buffers
Sanitization	200	3	0.5 N NaOH

Charge	200	3	0.250 M $NaPO_4$, pH 5.5
Equilibration	200	3-6	0.01M MES, 0.001M $NaPO_4$, 0.5M NaCl, pH 5.5
Wash 1	200	1	0.01M MES, 0.001M $NaPO_4$, 0.5M NaCl, pH 5.5
Wash 2	200	6	0.01M MES, 0.01M $NaPO_4$, 0.5M NaCl, pH 5.5
Elution	200	3	0.01M MES, 0.08M $NaPO_4$, pH 5.5
Strip	200	4	0.4M $NaPO_4$ pH 12
Clean	200	4	0.5 N NaOH
Store	200	4	0.1 N NaOH

Table 10. Exemplary Process Details for Phenyl Sepharose Chromatography

Process Step	Flow rate (cm/hr)	CV	Buffers
Sanitization	150	3	0.5 N NaOH
Equilibration	150	4-6	0.02 M MES, 1.5 M NaCl, pH 5.5
Wash	150	2	0.02 M MES, 1.5 M NaCl, pH 5.5
Elution	150	3	0.02 M MES, 0.2 M NaCl, pH 5.5
Water Wash	150	3	RO/DI Water
Ethanol Wash	150	3	20% Ethanol
Clean	150	3	0.5 N NaOH
Store	150	3	0.01 N NaOH

Table 11. Exemplary Diafiltration of the Phenyl Elution Pool

Filtration Unit	Centricon Plus 70
Diafiltration Buffer	0.02 M $NaPO_4$, 0.137 M NaCl, pH 6.0
Diafiltration Volumes	6X-8X

In Process Purity by HCP by ELISA

[0168] Table 12 describes the in-process HOP removal for each step. The in-process HCP results were high with the majority of removal at the HA step.

Table 12. In-Process HCP Removal

Step	HCP (ng/mg)	LRV	HCP Fold
Q	46,392	0.3	2
	51,957		
HA	51,957	1.3	18
	5,876		
Phenyl	5,876	0.7	5
	1,870		

Drug Substance Characterization

[0169] Exemplary drug substance lot release results are listed in Table 13. As can be seen, the drug substance had high specific activity and % FG in the purified material. Exemplary drug attributes characterization is shown in Table 13. HCP was reduced from 1,870 ng/mg to 372 ng/mg at the final UF/DF step.

Table 13. Exemplary Drug Substance Lot Release

DS Lot Release	1L CD media (I2S-AF)
%FG	94%
Glycan Map	
Group 3	99%
Group 5	89%
Group 6	104%
Group 7 (2-M6P)	95%
Total Area	107%
Sialic Acid	17
Internalization	83%
SEC-HPLC	99.9%
Specific Activity (U/mg)	82
IEX HPLC	
A (%)	64%
B (%)	23%
A+B	87%
E+F	0%
Host Cell Protein	372
Cell Uptake	98

Example 4. Physiochemical and Biological Characterization of Purified Recombinant I2S Enzyme

[0170] The purpose of the example was to perform a detailed characterization of the recombinant I2S protein purified using methods described above.

SDS-PAGE

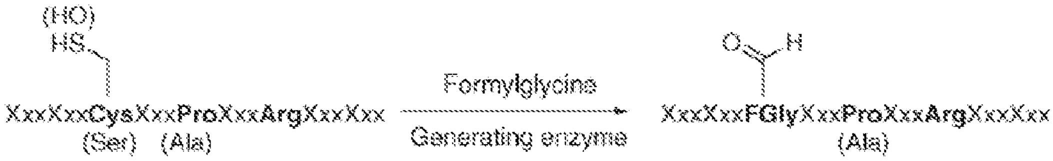
[0171] For the experiment, recombinant I2S protein was generated using the 2D and 4D human cell lines, in two separate serum-free cell culture reactions. Samples were collected and purified using methods described above. Purified I2S enzyme was analyzed by SDS-PAGE, and treated with silver stain for visualization. Exemplary results are shown in Figure 8. As can be seen from Figure 8, purified recombinant I2S protein using methods described herein present comparable banding patterns as compared to the I2S reference sample purified using standard method.

Peptide Map

[0172] Recombinant I2S protein produced by the I2S-AF 2D cell line was purified using methods as described above. Purified recombinant I2S and a sample of reference human I2S were each subjected to proteolytic digest and examined by HPLC analysis. An exemplary peptide map as compared to that of a reference I2S is shown in Figure 9.

Percent Formylglycine Conversion

[0173] Peptide mapping can be used to determine Percent FGly conversion. I2S activation requires Cysteine (corresponding to position 59 of the 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:

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

[0174] For example 50% FG means half of the purified recombinant I2S is enzymatically inactive without any therapeutic effect.

[0175] Peptide mapping was used to calculate %FG. Briefly, a purified 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 14.

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

[0176] The glycan and sialic acid composition of purified recombinant I2S protein was determined. Quantification of the glycan composition was performed, using anion exchange chromatography to produce a glycan map. As described below, the glycan map of recombinant I2S purified under conditions described herein 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 from the serum-free cell culture (I2S-AF 2D Serum-free and I2S-AF 4D Serum-free) and reference recombinant I2S, 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 ther 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 10, the glycan map for I2S purified from serum-free medium showed 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. Exemplary glycan mapsa are shown in Figure 10.

[0177] 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 visualized 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:

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

[0178] Exemplary data indicative of sialic acid content on the recombinant I2S purified from I2S-AF 2D or 4D cell lines are shown in Table 14.

Table 14: Exemplary Characteristics of I2S Purified from 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 (n=13)
Glycan Mapping	
Monosialylated	105
Disialylated	93
Monophosphorylated	139
Trisialylated	89
Tetrasialylated	125
Diphosphorylated	95
Sialic Acid (mol/mol)	20

Specific Activity

[0179] Specific activity of the recombinant I2S enzyme purified using methods described herein was analyzed using *in vitro* sulfate release assay or 4-MUF assay.

In vitro sulfate release assay

[0180] *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 14.

4-MUF assay

[0181] Specific activity of the purified recombinant I2S enzyme 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 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:

$$\%CV = \frac{S\tan dard\ Deviation\ of\ Raw\ Fluorescenc\ Values(N=3)}{Averagel\ Fluorescence\ Value} X100\%$$

[0182] 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:

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

CFU = Negative corrected average fluorescence

DF - Dilution Factor

[0183] 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

[0184] For this experiment, 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 showing charge profiles observed for recombinant I2S purified from 2D and 4D cell lines are shown in Figure 11.

What is claimed is:

1. A composition comprising purified recombinant iduronate-2-sulfatase (I2S) having an amino acid sequence at least 70% identical to SEQ ID NO:1,

wherein the purified recombinant I2S comprises at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly) and

further wherein the purified recombinant I2S contains less than 150 ng/mg Host Cell Protein (HCP).

2. The composition of claim 1, wherein the purified recombinant I2S contains less than 100 ng/mg HCP.

3. The composition of claim 1, wherein the purified recombinant I2S contains less than 80 ng/mg HCP.

4. The composition of claim 1, wherein the purified recombinant I2S contains less than 60 ng/mg HCP.

5. A composition comprising purified recombinant iduronate-2-sulfatase (I2S) having an amino acid sequence at least 70% identical to SEQ ID NO:1

wherein the purified recombinant I2S comprises at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly) and

further wherein the purified recombinant I2S contains, on average, at least 16 sialic acids per molecule.

6. The composition of claim 5, wherein the purified recombinant I2S contains, on average, at least 18 sialic acids per molecule.

7. The composition of claim 5, wherein the purified recombinant I2S contains, on average, at least 20 sialic acids per molecule.

8. A composition comprising purified recombinant iduronate-2-sulfatase (I2S) having an amino acid sequence at least 70% identical to SEQ ID NO:1

wherein the purified recombinant I2S comprises at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly) and

further wherein the purified recombinant I2S contains at least 10% bis-phosphorylated oligosaccharides per enzyme.

9. The composition of claim 8, wherein the purified recombinant I2S contains at least 20% bis-phosphorylated oligosaccharides per enzyme.

10. A composition comprising purified recombinant iduronate-2-sulfatase (I2S) having an amino acid sequence at least 70% identical to SEQ ID NO:1

wherein the purified recombinant I2S comprises at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly) and

further wherein the purified I2S is characterized with a glycan map comprising seven or fewer peak groups selected from the peak groups indicative of neutral (peak group 1), mono-sialylated (peak group 2), di-sialylated (peak group 3), monophosphorylated (peak group 4), tri-sialylated (peak group 5), tetra-sialylated (peak group 6), or diphosphorylated (peak group 7) I2S protein.

11. The composition of any one of the preceding claims, wherein the purified recombinant I2S comprises at least about 75% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

12. The composition of any one of claims 1-10, wherein the purified recombinant I2S comprises at least about 80% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

13. A composition comprising purified recombinant iduronate-2-sulfatase (I2S) having an amino acid sequence at least 70% identical to SEQ ID NO:1, wherein the purified recombinant I2S comprises greater than about 85% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

14. The composition of claim 13, wherein the purified recombinant I2S comprises greater than about 90% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

15. The composition of claim 13, wherein the purified recombinant I2S comprises greater than about 95% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

16. The composition of claim 13, wherein the purified recombinant I2S comprises substantially 100% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

17. The composition of any one of the preceding claims, wherein the purified recombinant I2S has an amino acid sequence at least 80% identical to SEQ ID NO:1.

18. The composition of any one of the preceding claims, wherein the purified recombinant I2S has an amino acid sequence at least 90% identical to SEQ ID NO:1.

19. The composition of any one of the preceding claims, wherein the purified recombinant I2S has an amino acid sequence at least 95% identical to SEQ ID NO:1.

20. The composition of any one of the preceding claims, wherein the purified recombinant I2S has an amino acid sequence identical to SEQ ID NO:1.

21. A formulation comprising the composition of any one of the preceding claims and a physiologically acceptable carrier.

22. The formulation of claim 21, wherein the formulation is suitable for intravenous administration.

23. The formulation of claim 21, wherein the formulation is suitable for intrathecal administration.

24. The formulation of claim 21, wherein the formulation is suitable for subcutaneous administration.

25. The formulation of claim 21, wherein the formulation is for treating Hunter syndrome.

26. A method comprising

purifying recombinant iduronate-2-sulfatase (I2S) protein from an impure preparation by conducting one or more of anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography, and

wherein the method involves less than 6 chromatography steps and wherein the purified recombinant I2S protein contains less than 100 ng/mg Host Cell Protein (HCP).

27. The method of claim 26, wherein the anion-exchange chromatography is Q chromatography.

28. The method of claim 26 or 27, wherein the cation-exchange chromatography is SP chromatography.

29. The method of any one of claims 26-28, wherein the mixed-mode chromatography is hydroxyapatite (HA) chromatography.

30. The method of any one of claims 26-29, wherein the hydrophobic interaction chromatography is phenyl chromatography.

31. The method of any one of claims 26-30, wherein the method involves 5 chromatography steps or less.

32. The method of any one of claims 26-31, wherein the method involves 4 chromatography steps or less.

33. The method of any one of claims 26-32, wherein the method conducts the anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography in that order.

34. The method of any one of claims 26-33, wherein the anion-exchange chromatography column, once loaded with the impure preparation or an intermediate eluate, is washed using a

wash buffer comprising NaCl at a concentration ranging between about 140 mM and 200 mM with pH 5.5.

35. The method of any one of claims 26-34, wherein the anion-exchange chromatography column is eluted using a elution buffer comprising a linear NaCl gradient.

36. The method of claim 33, wherein the linear NaCl gradient comprises a range from 0-500 mM NaCl.

37. The method of any one of claims 26-36, wherein the impure preparation or an intermediate eluate is adjusted to a conductivity ranging between about 1 mS/cm and 20 mS/cm prior to loading to the cation-exchange chromatography column.

38. The method of claim 37, wherein the cation-exchange chromatography column is run at a pH of about 5.5.

39. The method of any one of claims 26-38, wherein the impure preparation or an intermediate eluate is adjusted to phosphate concentration ranging from about 0.001-0.01 M and pH 5.5 prior to loading to the mixed-mode chromatography column.

40. The method of claim 39, wherein the mixed-mode chromatography column, once loaded, is washed using a wash buffer comprising phosphate concentration ranging between about 10-20 mM, pH 5.5.

41. The method of claim 40, wherein the mixed mode chromatography column is eluted using a elution buffer comprising phosphate concentration ranging between about 50-150 mM, pH 5.5.

42. The method of any one of claims 26-41, wherein the impure preparation or an intermediate eluate is adjusted to a salt concentration ranging from about 0.5-2.0 M, pH of about 4.5-6.0 prior to loading on to the hydrophobic interaction chromatography column.

43. The method of claim 42, wherein the hydrophobic interaction chromatography column, once loaded, is washed using a wash buffer comprising salt concentration ranging between about 0.5-2.0 M, pH of about 4.5-6.0.

44. The method of claim 43, wherein the hydrophobic interaction chromatography column is eluted using a elution buffer comprising salt concentration ranging between about 0.1-0.5 M, pH of about 4.5-6.0.

45. The method of any one of claims 26-44, wherein each of the anion-exchange chromatography, cation-exchange chromatography, mixed-mode chromatography, and hydrophobic interaction chromatography column has a height ranging from 14-25 cm.

46. The method of any one of the preceding claims, wherein the method further comprises a step of viral inactivation.

47. The method of claim 46, wherein the step of viral inactivation is before loading the impure preparation onto the first chromatography column.

48. The method of claim 46, wherein the step of viral inactivation comprises adding a detergent to the impure preparation.

49. The method of any one of claims 26-48, wherein the method further comprises a step of viral removal after the last Chromatography column.

50. The method of any one of claims 26-49, wherein the method further comprises a step of ultrafiltration and/or dialfiltration.

51. The method of claim 50, wherein the step of ultrafiltration and/or dialfiltration comprises exchanging the purified recombinant I2S protein into a drug formulation buffer.

52. The method of any one of claims 26-51, wherein the recombinant I2S protein has an amino acid sequence at least 70% identical to SEQ ID NO:1.

53. The method of any one of claims 26-52, wherein the recombinant I2S protein has an amino acid sequence identical to SEQ ID NO:1.

54. The method of any one of claims 26-53, wherein the recombinant I2S protein is produced by mammalian cells cultured in suspension in a serum-free medium.

55. The method of claim 54, wherein the mammalian cells are cultured in a bioreactor.

56. The method of claim 54 or 55, wherein the serum-free medium lacks animal-derived components.

57. The method of any one of claims 54-56, wherein the serum-free medium is a chemically-defined medium.

58. The method of any one of claims 54-56, wherein the mammalian cells co-express the recombinant I2S protein and formylglycine generating enzyme (FGE).

59. The method of claim 58, wherein the mammalian cells are human cells.

60. The method of any one of claims 54-59, wherein the impure preparation is prepared from the serum-free medium containing recombinant I2S protein secreted from the mammalian cells.

61. The method of claim 60, wherein the impure preparation is thawed from a frozen medium preparation.

62. The method of any one of claims 26-61, wherein the purified recombinant I2S protein contains less than 80 ng/mg HCP.

63. The method of any one of claims 26-62, wherein the purified recombinant I2S protein contains less than 60 ng/mg HCP.

64. The method of any one of claims 26-63, wherein the purified recombinant I2S protein contains, on average, 16-22 sialic acids per molecule.

65. The method of any one of claims 26-63, wherein the purified 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.

66. A pharmaceutical composition comprising a recombinant I2S protein purified according to a method of any one of claims 26-65.

67. A method of treating Hunter syndrome comprising administering into a subject in need of treatment a pharmaceutical composition of claim 66.

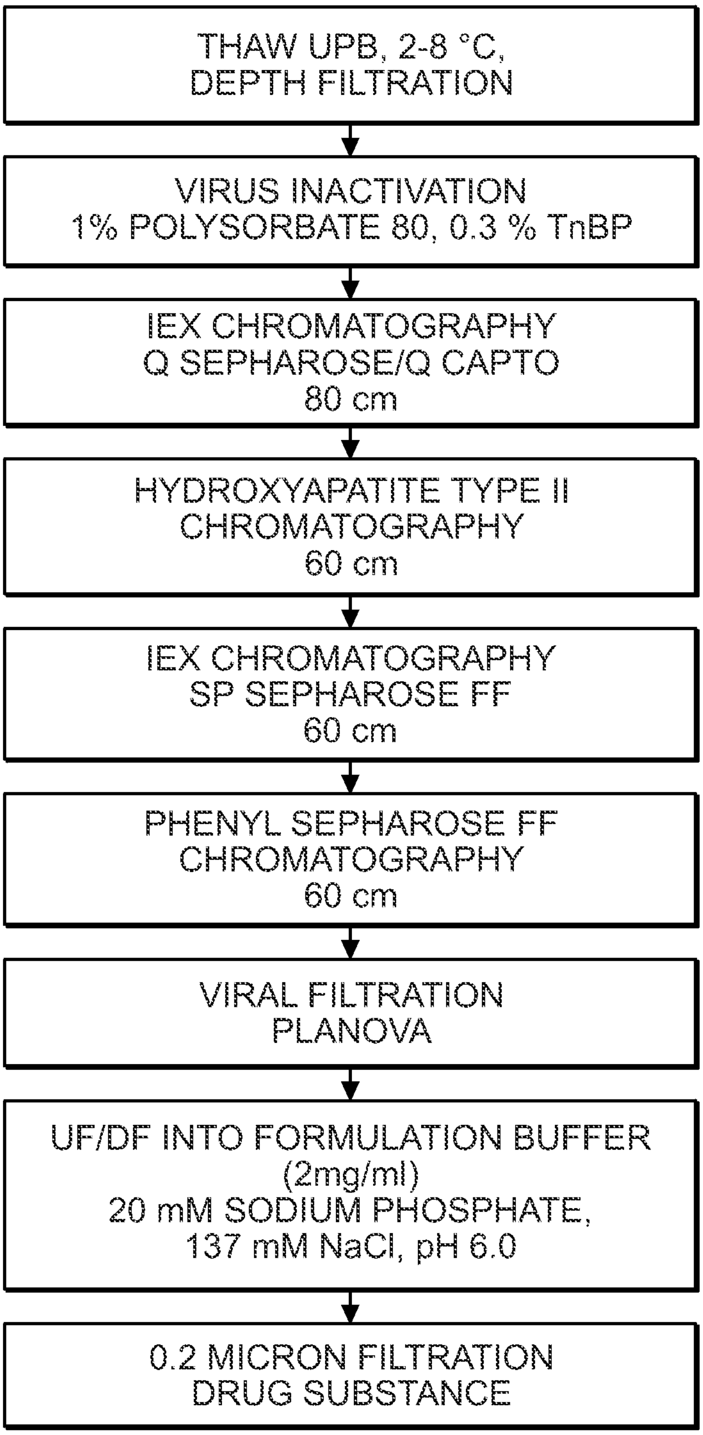

THAW UPB, 2-8 °C,
DEPTH FILTRATION
VIRUS INACTIVATION
1% POLYSORBATE 80, 0.3 % TnBP
IEX CHROMATOGRAPHY
Q SEPHAROSE/Q CAPTO
80 cm
HYDROXYAPATITE TYPE II
CHROMATOGRAPHY
60 cm
IEX CHROMATOGRAPHY
SP SEPHAROSE FF
60 cm
PHENYL SEPHAROSE FF
CHROMATOGRAPHY
60 cm
VIRAL FILTRATION
PLANOVA
UF/DF INTO FORMULATION BUFFER
(2mg/ml)
20 mM SODIUM PHOSPHATE,
137 mM NaCl, pH 6.0
0.2 MICRON FILTRATION
DRUG SUBSTANCE

FIG. 1

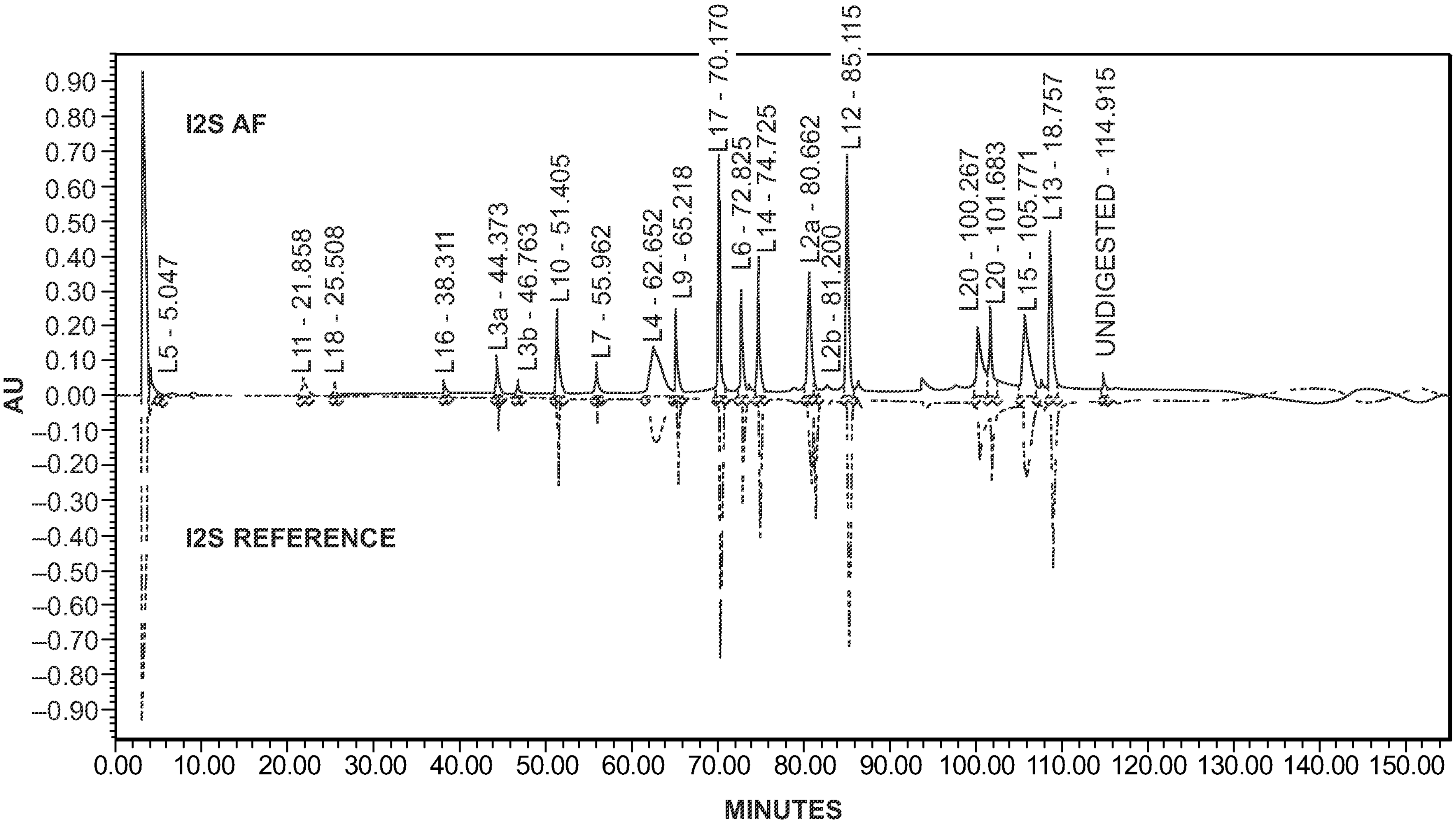
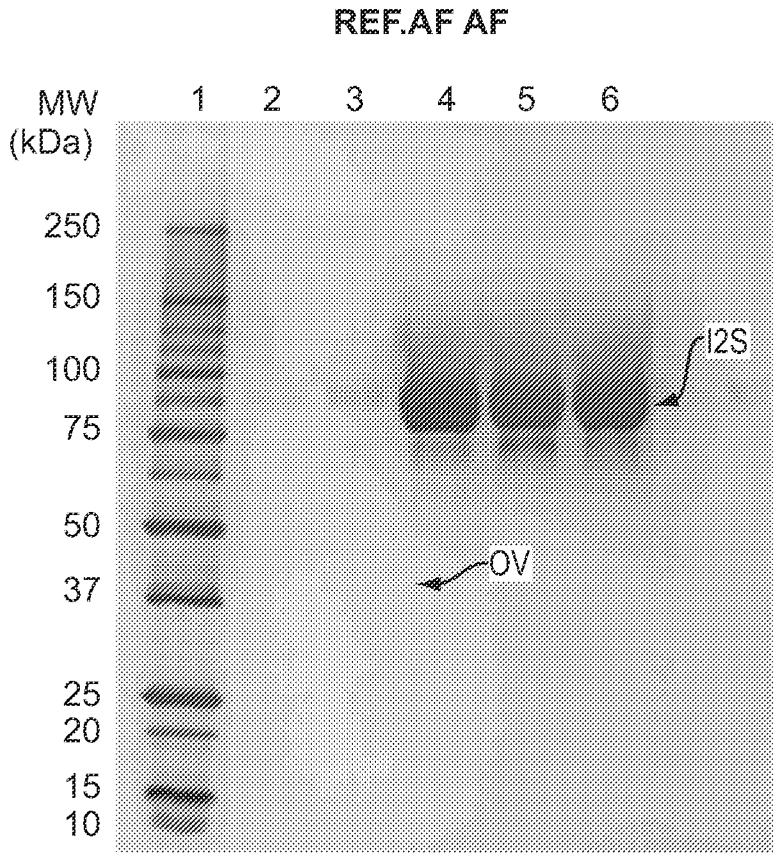

AU
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
–0.10
–0.20
–0.30
–0.40
–0.50
–0.60
–0.70
–0.80
–0.90
I2S AF
I2S REFERENCE
L5 - 5.047
L11 - 21.858
L18 - 25.508
L16 - 38.311
L3a - 44.373
L3b - 46.763
L10 - 51.405
L7 - 55.962
L4 - 62.652
L9 - 65.218
L17 - 70.170
L6 - 72.825
L14 - 74.725
L2a - 80.662
L2b - 81.200
L12 - 85.115
L20 - 100.267
L20 - 101.683
L15 - 105.771
L13 - 18.757
UNDIGESTED - 114.915
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
150.00
MINUTES

FIG. 2



LANE	*LOAD SAMPLE*	*PAD ID*	*LOAD VOL. µl*	*TOTAL* µg
1	PRECISION PLUS PROTEIN STDS (UNSTAINED)	▨	3 µl	▨
2	ASSAY CONTROL #1	▨	20	8 µg EACH, OV END REF
3	ASSAY CONTROL #2	▨	20	18 µg EACH, OV END REF
4	REFERENCE STANDARD	▨	20	8
5	RDSF 11-101 MOCK DS	11AD0545-1	20	8
6	RDSF 11-105 MOCK DS	11AD0545-2	20	8

FIG. 3

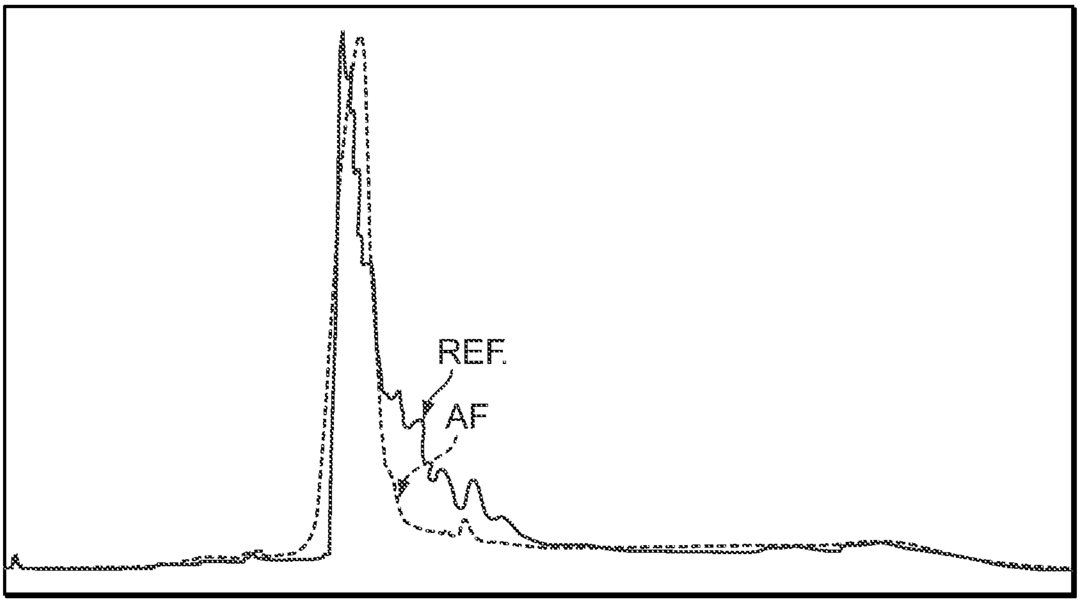


FIG. 4

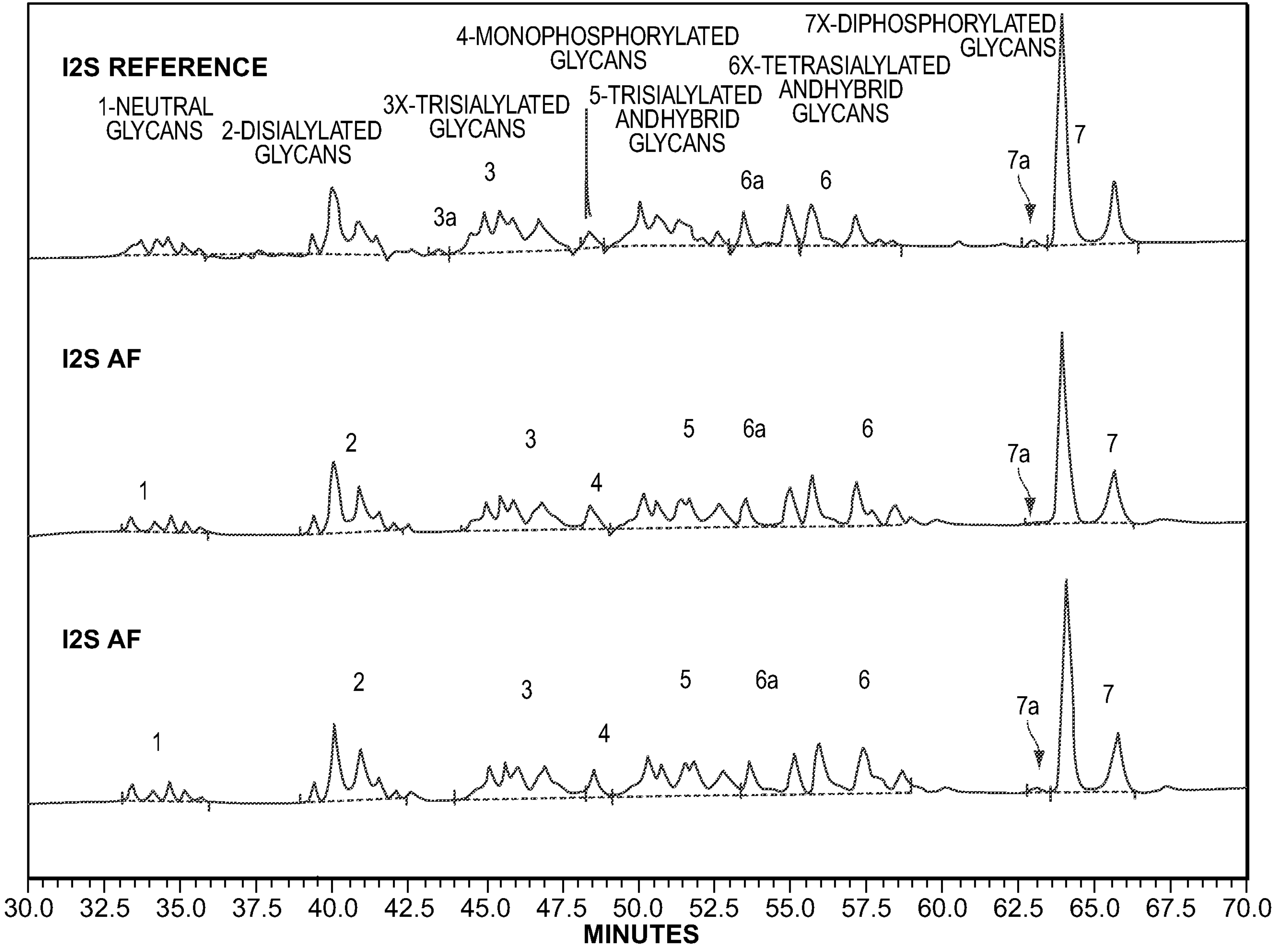

I2S REFERENCE
1-NEUTRAL GLYCANS
2-DISIALYLATED GLYCANS
3X-TRISIALYLATED GLYCANS
4-MONOPHOSPHORYLATED GLYCANS
5-TRISIALYLATED ANDHYBRID GLYCANS
6X-TETRASIALYLATED ANDHYBRID GLYCANS
7X-DIPHOSPHORYLATED GLYCANS
3a
3
6a
6
7a
7
I2S AF
1
2
3
4
5
6a
6
7a
7
I2S AF
1
2
3
4
5
6a
6
7a
7
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
70.0
MINUTES

FIG. 5

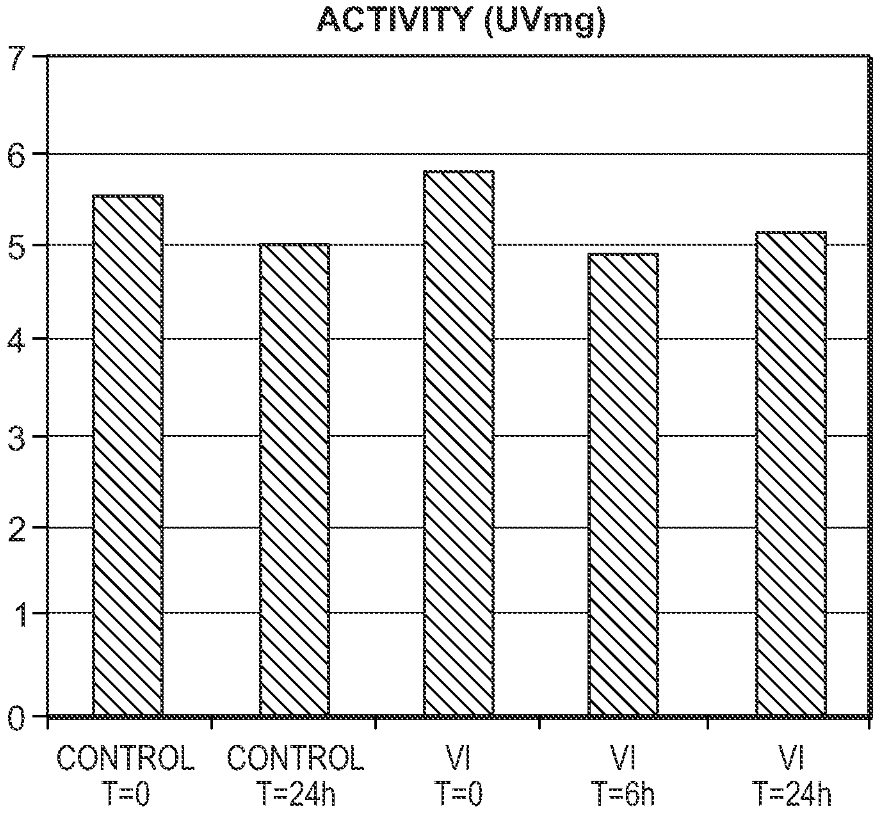

ACTIVITY (UVmg)
7
6
5
4
3
2
1
0
CONTROL T=0
CONTROL T=24h
VI T=0
VI T=6h
VI T=24h

FIG. 6

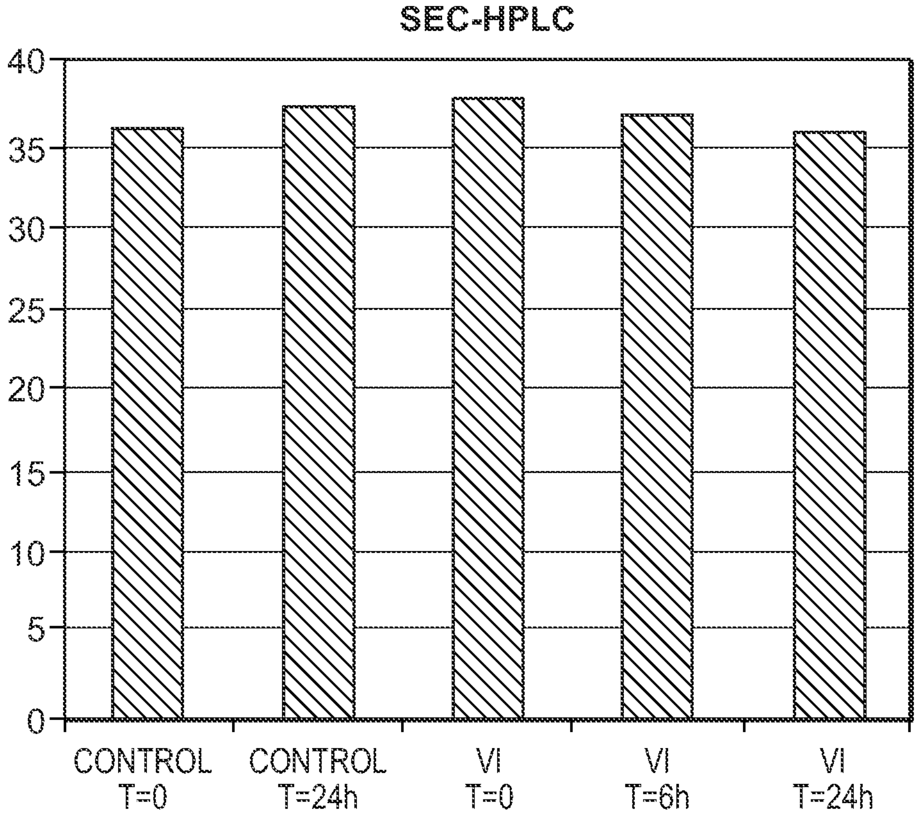
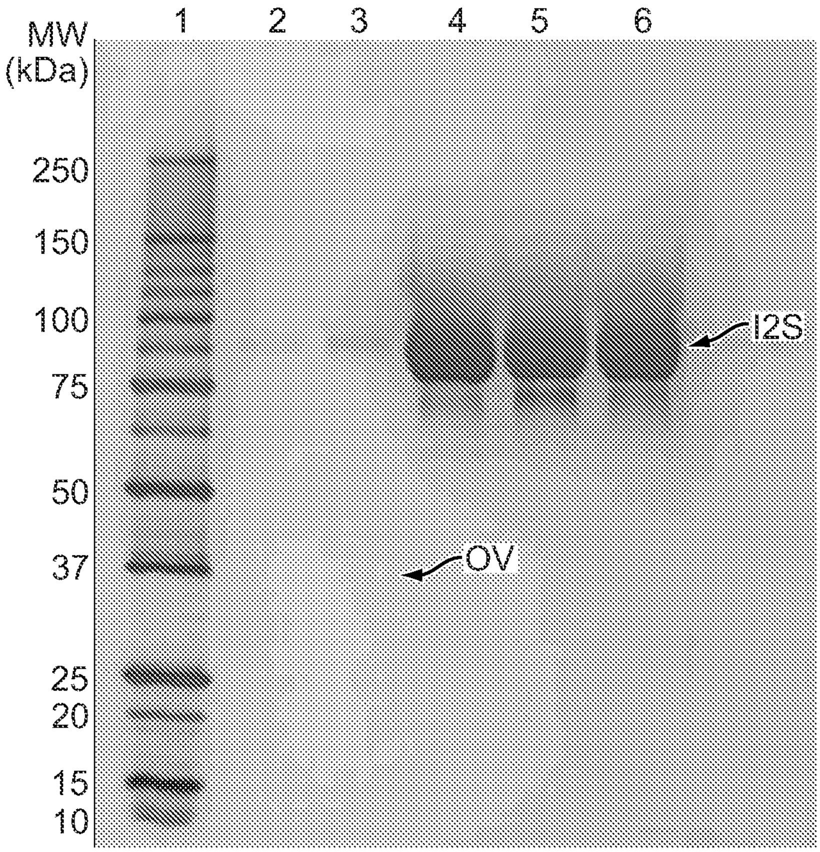

SEC-HPLC
40
35
30
25
20
15
10
5
0
CONTROL T=0
CONTROL T=24h
VI T=0
VI T=6h
VI T=24h

FIG. 7



LANE	LOAD SAMPLE	LOAD VOLUME	TOTAL µg
1	PROTEIN STDS	3 µl	
2	ASSAY CONTROL #18	20 µl	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. 8

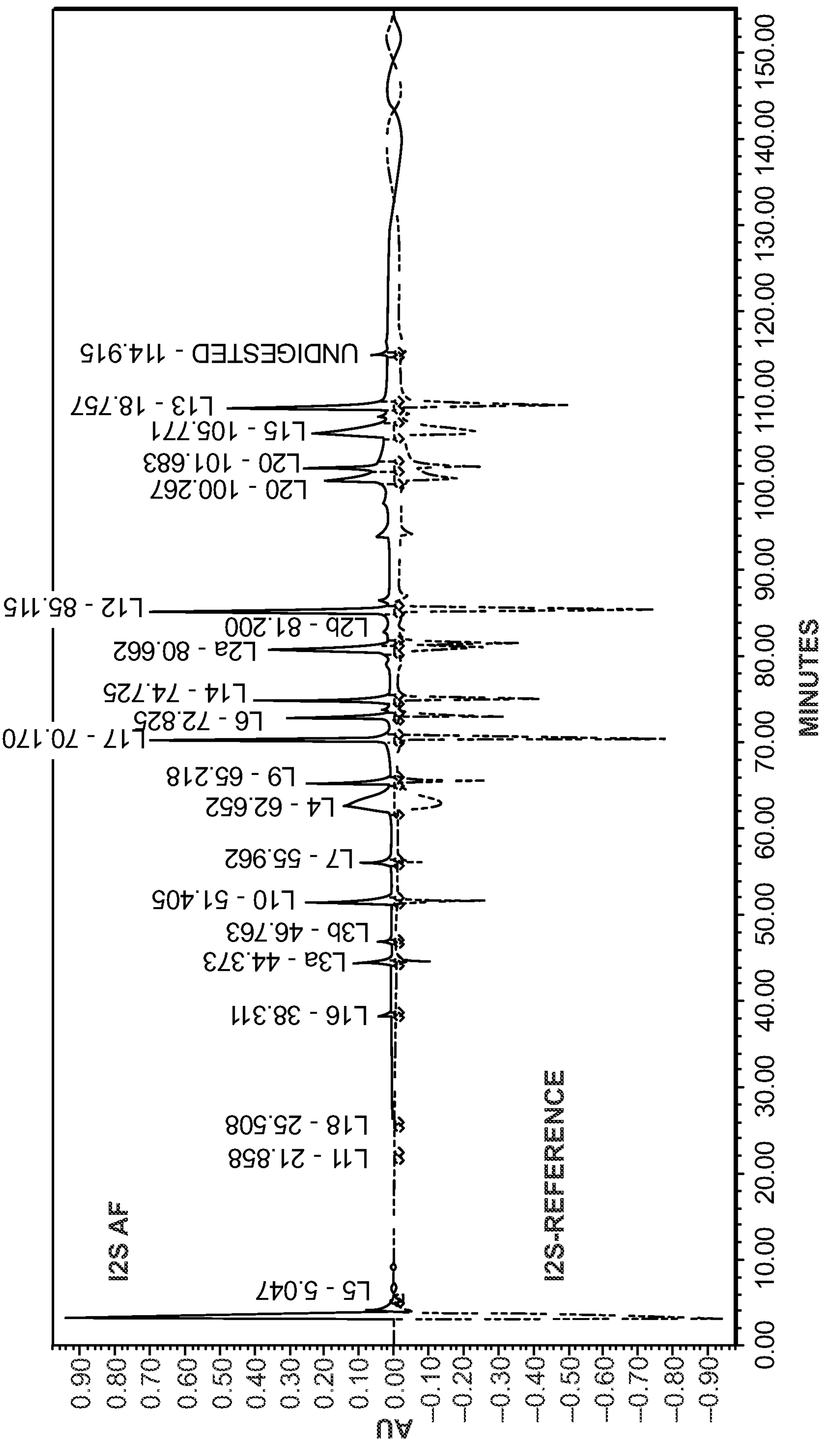

I2S AF
I2S-REFERENCE
L5 - 5.047
L11 - 21.858
L18 - 25.508
L16 - 38.311
L3a - 44.373
L3b - 46.763
L10 - 51.405
L7 - 55.962
L4 - 62.652
L9 - 65.218
L17 - 70.170
L6 - 72.825
L14 - 74.725
L2a - 80.662
L2b - 81.200
L12 - 85.115
L20 - 100.267
L20 - 101.683
L15 - 105.771
L13 - 18.757
UNDIGESTED - 114.915
AU
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
−0.10
−0.20
−0.30
−0.40
−0.50
−0.60
−0.70
−0.80
−0.90
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
150.00
MINUTES

FIG. 9

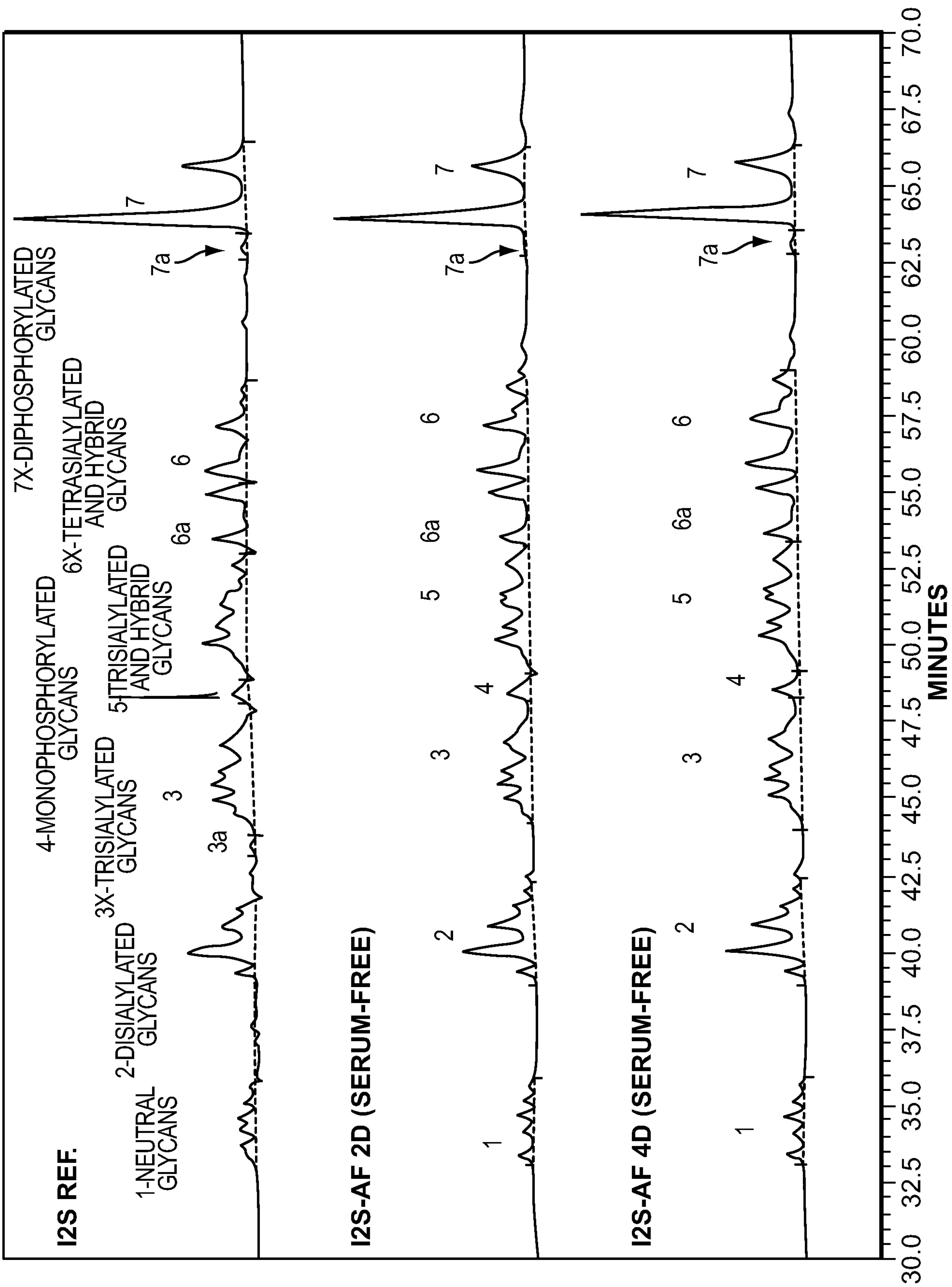

I2S REF.
1-NEUTRAL GLYCANS
2-DISIALYLATED GLYCANS
3X-TRISIALYLATED GLYCANS
4-MONOPHOSPHORYLATED GLYCANS
5-TRISIALYLATED AND HYBRID GLYCANS
6X-TETRASIALYLATED AND HYBRID GLYCANS
7X-DIPHOSPHORYLATED GLYCANS
3a
3
6a
6
7a
7
I2S-AF 2D (SERUM-FREE)
1
2
3
4
5
6a
6
7a
7
I2S-AF 4D (SERUM-FREE)
1
2
3
4
5
6a
6
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
MINUTES

FIG. 10

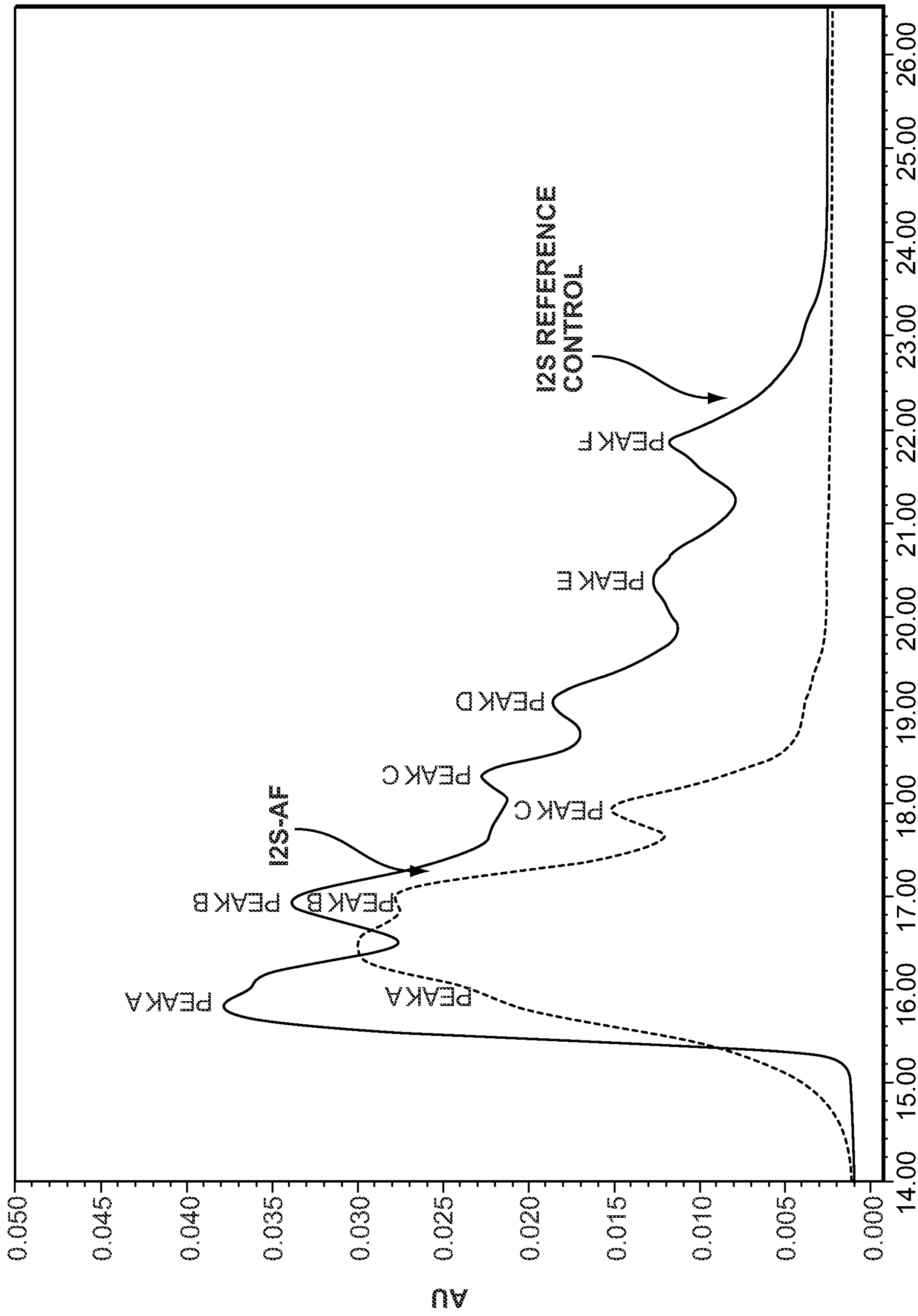

AU
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
22.00
23.00
24.00
25.00
26.00
MINUTES
PEAK A
PEAK B
PEAK C
PEAK D
PEAK E
PEAK F
PEAK A
PEAK B
PEAK C
I2S-AF
I2S REFERENCE CONTROL

FIG. 11

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

(19) World Intellectual Property Organization
International Bureau

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WIPO | PCT

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C07K 1/00 (2006.01) ***A61K 38/17*** (2006.01)
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(71) Applicant: SHIRE HUMAN GENETIC THERAPIES, INC. [US/US]; 300 Shire Way, Lexington, Massachusetts 02421 (US).

(72) Inventor: NICHOLS, Dave; 300 Shire Way, Lexington, Massachusetts 02421 (US).

(74) Agent: HUDDLESON, Justin P.; Choate, Hall & Stewart LLP, Two International Place, Boston, Massachusetts 02110 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR,

[Continued on next page]

(54) Title: PURIFICATION OF IDURONATE-2-SULFATASE

(57) Abstract: The present invention provides, among other things, improved methods for purifying 12 S protein produced recombinantly for enzyme replacement therapy. The present invention is, in part, based on the surprising discovery that recombinant I2S protein can be purified from unprocessed biological materials, such as, I2S-containing cell culture medium, using a process involving as few as four chromatography columns.

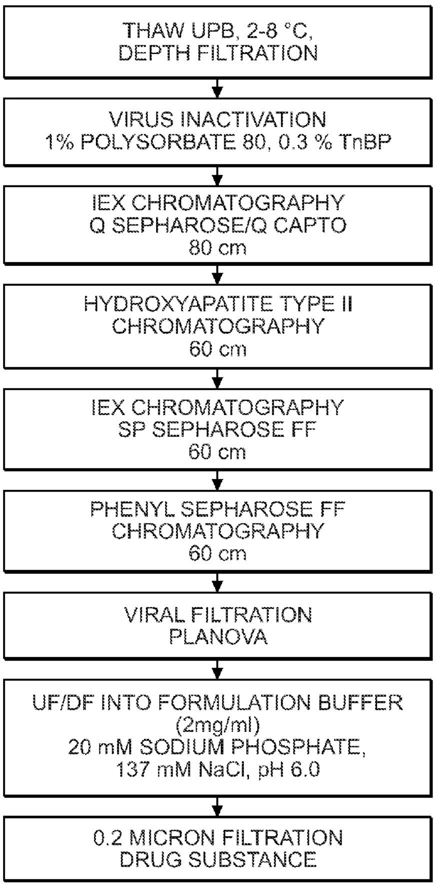

FIG. 1

KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report: 27 February 2014

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/48561

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 1/00, 16/00; A61K 38/17 (2013.01)
USPC - 530/412, 413, 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): A23J 1/00; C07K 1/00, 14/00, 16/00, 17/00; A61L 33/00; A61K 38/46, 38/17; A61P 31/12; C12Q 1/68; C12N 9/16 (2013.01)
USPC: 530/413, 412, 350, 387.1; 522/87; 436/6; 435/69.1, 196, 320.1, 325; 536/23.2

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; ProQuest; ScienceDirect; 'iduronate sulfatase,' formylglyceine, enzyme, cysteine, 'host cell protein',' glycan, sialic, phosphorylated, chromatography, purified, recombinant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 8128925 B2 (VELLARD, MC et al.) March 6, 2012; column 1, lines 14-25; column 3, lines 39-49; column 5, lines 17-22; column 6, lines 11-33; column 8, lines 25-43; column 12, lines 62-67; column 13, lines 1-22	1-16, 26-28
Y	WO 2011/044542 A1 (PARDRIDGE, WM et al.) April 14, 2011; paragraphs [0001], [0003], [0004], [0022], [0051], [0099], [00161], [00164]-[00166], [00183]	1-16
Y	US 7323553 B2 (FAHRNER, R et al.) January 29, 2008; column 9, lines 61-67; column 10, lines 9-17; column 42, lines 13-47 to column 43, lines 1-28; column 44, lines 31-34	1-12, 26-28
Y	US 7541164 B2 (SCHILLING, BM et al.) June 2, 2009; column 1, lines 40-45; column 8, lines 61-67; column 24, lines 12-15; column 38, lines 48-52; column 39, lines 32-45; column 40, lines 44-47; columns 41-42, Table 3	5-7, 11/5, 11/6, 11/7, 12/5, 12/6, 12/7
Y	US 6096555 A (HERMENTIN, P et al.) August 01, 2000; column 2, lines 8-13; column 4, lines 3-34	10, 11/10, 12/10

☐ 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 27 November 2013 (27.11.2013)	Date of mailing of the international search report 12 DEC 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/48561

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.: 17-25, 29-67
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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代理人 邬玥 葛强

(51)Int.Cl.
C07K 1/00(2006.01)
C07K 16/00(2006.01)
A61K 38/17(2006.01)

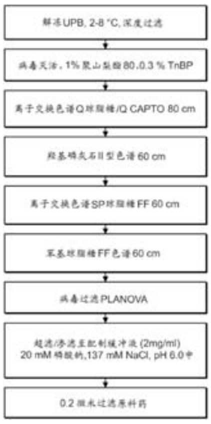
权利要求书4页 说明书36页
序列表11页 附图10页

(54) 发明名称

艾杜糖-2-硫酸酯酶的纯化

(57) 摘要

本发明除其它事项以外提供了用于纯化重组产生以用于酶替代疗法的I2S蛋白的改进方法。本发明部分地基于以下令人惊讶的发现，即可使用包括少至4个色谱柱的工艺从未经处理的生物材料例如含I2S的细胞培养基纯化重组I2S蛋白。



1.一种包含纯化的重组艾杜-2-硫酸酯酶(I2S)的组合物,所述重组艾杜糖-2-硫酸酯酶具有与SEQ ID NO:1具有至少70%的同一性的氨基酸序列,

其中所述纯化的重组I2S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Cα-甲酰甘氨酸(FGly)的转化和

另外地其中所述纯化的重组I2S含有小于150ng/mg的宿主细胞蛋白质(HCP)。

2.根据权利要求1所述的组合物,其中所述纯化的重组I2S含有小于100ng/mg的HCP。

3.根据权利要求1所述的组合物,其中所述纯化的重组I2S含有小于80ng/mg的HCP。

4.根据权利要求1所述的组合物,其中所述纯化的重组I2S含有小于60ng/mg的HCP。

5.一种包含纯化的重组艾杜糖-2-硫酸酯酶(I2S)的组合物,所述重组艾杜糖-2-硫酸酯酶具有与SEQ ID NO:1具有至少70%的同一性的氨基酸序列

其中所述纯化的重组I2S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Cα-甲酰甘氨酸(FGly)的转化和

另外地其中所述纯化的重组I2S含有平均每分子至少16个唾液酸。

6.根据权利要求5所述的组合物,其中所述纯化的重组I2S含有平均每分子至少18个唾液酸。

7.根据权利要求5所述的组合物,其中所述纯化的重组I2S含有平均每分子至少20个唾液酸。

8.一种包含纯化的重组艾杜糖-2-硫酸酯酶(I2S)的组合物,所述重组艾杜糖-2-硫酸酯酶具有与SEQ ID NO:1具有至少70%的同一性的氨基酸序列

其中所述纯化的重组I2S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Cα-甲酰甘氨酸(FGly)的转化和

另外地其中所述纯化的重组I2S含有每种酶至少10%的二磷酸化寡糖。

9.根据权利要求8所述的组合物,其中所述纯化的重组I2S含有每种酶至少20%的二磷酸化寡糖。

10.一种包含纯化的重组艾杜糖-2-硫酸酯酶(I2S)的组合物,所述重组艾杜糖-2-硫酸酯酶具有与SEQ ID NO:1具有至少70%的同一性的氨基酸序列

其中所述纯化的重组I2S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Cα-甲酰甘氨酸(FGly)的转化和

另外地其中所述纯化的I2S的特征在于包含7个或更少的峰组的聚糖图谱,所述峰组选自指示中性(峰组1)、单唾液酸化(峰组2)、二唾液酸化(峰组3)、单磷酸化(峰组4)、三唾液酸化(峰组5)、四唾液酸化(峰组6)或二磷酸化(峰组7)I2S蛋白的峰组。

11.根据前述权利要求中任一项所述的组合物,其中所述纯化的重组I2S包含至少约75%的对应于SEQ ID NO:1的Cys59的所述半胱氨酸残基至Cα-甲酰甘氨酸(FGly)的转化。

12.根据权利要求1-10中任一项所述的组合物,其中所述纯化的重组I2S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Cα-甲酰甘氨酸(FGly)的转化。

13.一种包含纯化的重组艾杜糖-2-硫酸酯酶(I2S)的组合物,所述重组艾杜糖-2-硫酸酯酶具有与SEQ ID NO:1具有至少70%的同一性的氨基酸序列,其中所述纯化的重组

I2S 包含大于约 85％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 Cα－甲酰甘氨酸（FGly）的转化。

14. 根据权利要求 13 所述的组合物，其中所述纯化的重组 I2S 包含大于约 90％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 Cα－甲酰甘氨酸（FGly）的转化。

15. 根据权利要求 13 所述的组合物，其中所述纯化的重组 I2S 包含大于约 95％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 Cα－甲酰甘氨酸（FGly）的转化。

16. 根据权利要求 13 所述的组合物，其中所述纯化的重组 I2S 包含大体上 100％的对应于 SEQ ID NO:1 的 Cys59 的所述半胱氨酸残基至 Cα－甲酰甘氨酸（FGly）的转化。

17. 根据前述权利要求中任一项所述的组合物，其中所述纯化的重组 I2S 具有与 SEQ ID NO:1 具有至少 80％的同一性的氨基酸序列。

18. 根据前述权利要求中任一项所述的组合物，其中所述纯化的重组 I2S 具有与 SEQ ID NO:1 具有至少 90％的同一性的氨基酸序列。

19. 根据前述权利要求中任一项所述的组合物，其中所述纯化的重组 I2S 具有与 SEQ ID NO:1 具有至少 95％的同一性的氨基酸序列。

20. 根据前述权利要求中任一项所述的组合物，其中所述纯化的重组 I2S 具有与 SEQ ID NO:1 相同的氨基酸序列。

21. 一种制剂，其包含根据前述权利要求中任一项所述的组合物和生理上可接受的载体。

22. 根据权利要求 21 所述的制剂，其中所述制剂适合用于静脉内施用。

23. 根据权利要求 21 所述的制剂，其中所述制剂适合用于鞘内施用。

24. 根据权利要求 21 所述的制剂，其中所述制剂适合用于皮下施用。

25. 根据权利要求 21 所述的制剂，其中所述制剂用于治疗亨特综合症。

26. 一种方法，其包括

通过进行阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱中的一种或多种从不纯制剂纯化重组艾杜糖 -2- 硫酸酯酶（I2S）蛋白，和

其中所述方法包括少于 6 个色谱步骤并且其中所述纯化的重组 I2S 蛋白含有小于 100ng/mg 的宿主细胞蛋白质（HCP）。

27. 根据权利要求 26 所述的方法，其中所述阴离子交换色谱为 Q 色谱。

28. 根据权利要求 26 或 27 所述的方法，其中所述阳离子交换色谱为 SP 色谱。

29. 根据权利要求 26-28 中任一项所述的方法，其中所述混合模式色谱为羟基磷灰石（HA）色谱。

30. 根据权利要求 26-29 中任一项所述的方法，其中所述疏水相互作用色谱为苯基色谱。

31. 根据权利要求 26-30 中任一项所述的方法，其中所述方法包括 5 个色谱步骤或更少色谱步骤。

32. 根据权利要求 26-31 中任一项所述的方法，其中所述方法包括 4 个色谱步骤或更少色谱步骤。

33. 根据权利要求 26-32 中任一项所述的方法，其中所述方法按照该顺序进行阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱。

34.根据权利要求26-33中任一项所述的方法，其中在上样不纯制剂或中间洗脱液后，使用pH 5.5的包含浓度在约140mM和200mM之间的范围内的NaCl的洗涤缓冲液洗涤所述阴离子交换色谱柱。

35.根据权利要求26-34中任一项所述的方法，其中使用包含线性NaCl梯度的洗脱缓冲液洗脱所述阴离子交换色谱柱。

36.根据权利要求33所述的方法，其中所述线性NaCl梯度包含在0-500mM范围内的NaCl。

37.根据权利要求26-36中任一项所述的方法，其中在上样至所述阳离子交换色谱柱之前，将所述不纯制剂或中间洗脱液调整至在约1mS/cm和20mS/cm之间的范围内的电导率。

38.根据权利要求37所述的方法，其中在约5.5的pH下运行所述阳离子交换色谱柱。

39.根据权利要求26-38中任一项所述的方法，其中在上样至所述混合模式色谱柱之前，将所述不纯制剂或中间洗脱液调整至在约0.001-0.01M的范围内的磷酸盐浓度和pH 5.5。

40.根据权利要求39所述的方法，其中在上样后，使用pH 5.5的包含在约10-20mM的范围内的磷酸盐浓度的洗涤缓冲液洗涤所述混合模式色谱柱。

41.根据权利要求40所述的方法，其中使用pH 5.5的包含在约50-150mM的范围内的磷酸盐浓度的洗脱缓冲液洗脱所述混合模式色谱柱。

42.根据权利要求26-41中任一项所述的方法，其中在上样至疏水相互作用色谱柱上之前，将所述不纯制剂或中间洗脱液调整至的在约0.5-2.0M的范围内的盐浓度，pH为约4.5-6.0。

43.根据权利要求42所述的方法，其中在上样后，使用pH为约4.5-6.0的包含在约0.5-2.0M的范围内的盐浓度的洗涤缓冲液洗涤所述疏水相互作用色谱柱。

44.根据权利要求43所述的方法，其中使用pH为约4.5-6.0的包含在约0.1-0.5M的范围内的盐浓度的洗脱缓冲液洗脱所述疏水相互作用色谱柱。

45.根据权利要求26-44中任一项所述的方法，其中所述阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱柱中的每一种具有在14-25cm的范围内的高度。

46.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病毒灭活的步骤。

47.根据权利要求46所述的方法，其中所述病毒灭活的步骤在将所述不纯制剂上样至所述第一色谱柱上之前进行。

48.根据权利要求46所述的方法，其中所述病毒灭活的步骤包括将去垢剂添加至所述不纯制剂。

49.根据权利要求26-48中任一项所述的方法，其中所述方法还包括在所述最后一个色谱柱之后除去病毒的步骤。

50.根据权利要求26-49中任一项所述的方法，其中所述方法还包括超滤和/或渗滤的步骤。

51.根据权利要求50所述的方法，其中所述超滤和/或渗滤的步骤包括将所述纯化的重组I2S蛋白交换至药物配制缓冲液中。

52.根据权利要求26-51中任一项所述的方法，其中所述重组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。

53.根据权利要求26-52中任一项所述的方法，其中所述重组I2S蛋白具有与SEQ ID NO:1相同的氨基酸序列。

54.根据权利要求26-53中任一项所述的方法，其中所述重组I2S蛋白由在无血清培养基中悬浮培养的哺乳动物细胞产生。

55.根据权利要求54所述的方法，其中在生物反应器中培养所述哺乳动物细胞。

56.根据权利要求54或55所述的方法，其中所述无血清培养基缺乏动物来源的组分。

57.根据权利要求54-56中任一项所述的方法，其中所述无血清培养基是化学成分确定的培养基。

58.根据权利要求54-56中任一项所述的方法，其中所述哺乳动物细胞共表达所述重组I2S蛋白和甲酰甘氨酸生成酶(FGE)。

59.根据权利要求58所述的方法，其中所述哺乳动物细胞为人细胞。

60.根据权利要求54-59中任一项所述的方法，其中从含有由所述哺乳动物细胞分泌的重组I2S蛋白的所述无血清培养基制备所述不纯制剂。

61.根据权利要求60所述的方法，其中将所述不纯制剂从冷冻的培养基制剂解冻。

62.根据权利要求26-61中任一项所述的方法，其中所述纯化的重组I2S蛋白含有小于80ng/mg的HCP。

63.根据权利要求26-62中任一项所述的方法，其中所述纯化的重组I2S蛋白含有小于60ng/mg的HCP。

64.根据权利要求26-63中任一项所述的方法，其中所述纯化的重组I2S蛋白含有平均每分子16-22个唾液酸。

65.根据权利要求26-63中任一项所述的方法，其中所述纯化的重组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。

66.一种药物组合物，其包含根据如权利要求26-65中任一项所述的方法纯化的重组I2S蛋白。

67.一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66所述的药物组合物。

艾杜糖-2-硫酸酯酶的纯化

[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61/666,733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“2006685-0342_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15KB。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纯化重组产生以用于酶替代疗法的I2S蛋白的改进方法。本发明部分地基于以下令人惊讶的发现,即可使用包括少至4个色谱柱的工艺从未经处理的生物材料例如含I2S的细胞培养基纯化重组I2S蛋白。用于酶替代疗法的重组I2S的批准现有的纯化工艺包括6个色谱柱。如在实施例部分中描述的,使用根据本发明的4柱工艺纯化的重组I2S蛋白符合美国和许多其他国家的市场营销纯度要求。此外,根据本发明纯化的重组I2S酶保留高百分比的C_{α}-甲酰甘氨酸(FGly)(例如,高于70%并高达100%)(这对于I2S酶的活性是重要的)和明显的特性例如唾液酸含量和聚糖图谱(这可促进重组I2S蛋白的生物利用度和/或溶酶体靶向)。因此,本发明提供了有效的、更廉价且更快速的用于纯化重组I2S蛋白的工艺。本发明特别适用于纯化在无血清培养基中产生的重组I2S蛋白。

[0011] 因此,在一个方面,本发明提供了使用基于阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱中的一种或多种的工艺从不纯制剂纯化重组I2S蛋白的

方法。在一些实施方案中，根据本发明的发明方法包括少于 6 个（例如，少于 5 个，少于 4 个或少于 3 个）色谱步骤。在一些实施方案中，根据本发明的发明方法包括 2、3、4 或 5 个色谱步骤。在一些实施方案中，根据本发明的发明方法包括 4 个色谱步骤。在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白含有小于 100ng/mg 的宿主细胞蛋白质 (HCP)（例如，小于 90ng/mg 的 HCP、小于 80ng/mg 的 HCP、小于 70ng/mg 的 HCP、小于 60ng/mg 的 HCP、小于 50ng/mg 的 HCP、小于 40ng/mg 的 HCP、小于 30ng/mg 的 HCP、小于 20ng/mg 的 HCP、小于 10ng/mg 的 HCP）。

[0012]　在一些实施方案中，合适的阴离子交换色谱为 Q 色谱。在一些实施方案中，合适的阳离子交换色谱为 SP 色谱。在一些实施方案中，适合的混合模式色谱为羟基磷灰石 (HA) 色谱。在一些实施方案中，合适的疏水相互作用色谱为苯基色谱。

[0013]　预期阴离子交换色谱（例如，Q 柱）、阳离子交换色谱（例如，SP 柱）、混合模式色谱（例如，HA 柱）以及疏水相互作用色谱（例如，苯基柱）可以以任何顺序进行。在一些实施方案中，根据本发明的方法按照该顺序进行阴离子交换色谱（例如，Q 柱）、阳离子交换色谱（例如，SP 柱）、混合模式色谱（例如，HA 柱）以及疏水相互作用色谱（例如，苯基柱）。

[0014]　在一些实施方案中，在上样至阴离子交换色谱柱（例如，Q 柱）之前，将不纯制剂或中间洗脱液或过柱液 (flow-through) 调整至约 5.0-7.0（例如，约 5.0、5.5、6.0、6.5 或 7.0) 的 pH 以及约 10-20mS/cm（例如，10、11、12、13、14、15、16、17、18、19 或 20mS/cm) 的电导率。在一些实施方案中，使用 1M 醋酸钠调整 pH。在一些实施方案中，使用 5M 氯化钠调整电导率。在一些实施方案中，上样后，使用 pH 为约 5.0-7.0（例如，约 5.0、5.5、6.0、6.5 或 7.0) 的包含在约 140mM 至 200mM（例如，约 140mM、145mM、150mM、155mM、160mM、165mM、170mM、175mM、180mM、185mM、190mM、195mM 或 200mM) 的范围内的盐（例如，NaCl) 浓度的洗涤缓冲液洗涤阴离子交换色谱柱。在一些实施方案中，使用包含线性盐（例如，NaCl) 梯度的洗脱缓冲液洗脱阴离子交换色谱柱。在一些实施方案中，适当的线性 NaCl 梯度含有在约 0-500mM 范围内的 NaCl（例如，约 0-400mM、约 0-350mM、约 0-300mM、约 50-500mM、约 150-500mM、约 150-450mM、约 150-400mM)。

[0015]　在一些实施方案中，在上样至阳离子交换色谱柱（例如，SP 柱）之前，将不纯制剂或中间洗脱液或过柱液调整至在约 1mS/cm 与 20mS/cm 之间（例如，约 1mS/cm 与 15mS/cm 之间、约 1mS/cm 与 10mS/cm 之间、约 1mS/cm 与 8mS/cm 之间、约 1mS/cm 与 6mS/cm 之间、约 1mS/cm 与 4mS/cm 之间、约 2mS/cm 与 4mS/cm) 的范围内的电导率。在一些实施方案中，在上样至阳离子交换色谱柱（例如，SP 柱）之前，将不纯制剂或中间洗脱液或过柱液调整至在约 2mS/cm 与 4mS/cm 之间（例如，2、2.5、3、3.5 或 4mS/cm) 的范围内的电导率。在一些实施方案中，通过用 H_2O 以约 1-2:1（例如，1:1、1.1:1、1.2:1、1.3:1、1.4:、1.5:1、1.6:1、1.7:1、1.8:1、1.9:1 或 2:1) 的比率从阴离子交换色谱柱稀释洗脱液来调整电导率。在一些实施方案中，通过渗滤调整电导率。在一些实施方案中，在约 5.0-6.5（例如，约 5.0、5.5、6.0 或 6.5) 的 pH 下运行阳离子交换色谱柱。在一些实施方案中，利用包含在约 0.01M 至约 0.1M（例如，约 0.01M、0.02M、0.03M、0.04M、0.05M、0.06M、0.07M、0.08M、0.09M 或 0.1M) 的范围内的磷酸盐（例如，NaPO4) 浓度的缓冲液运行阳离子交换色谱柱。在一些实施方案中，合适的 pH 为约 5.0-6.5（例如，约 5.0、5.5、6.0 或 6.5)。

[0016]　在一些实施方案中，在上样至混合模式色谱柱（例如，HA 柱）之前，将不纯制剂

或中间洗脱液或过柱液调整至在约 0.001M 至约 0.01M(例如,约 0.001M、0.002M、0.003M、0.004M、0.005M、0.006M、0.007M、0.008M、0.009M 或 0.01M)的范围内的磷酸盐(例如,NaPO4)浓度和约 5.0-6.5(例如,约 5.0、5.5、6.0 或 6.5)的 pH。在一些实施方案中,在上样后,用中性 pH 或接近中性 pH 的含有磷酸盐(例如,1-10mM 的磷酸钠或磷酸钾)的洗涤缓冲液来洗涤混合模式色谱柱(例如,HA 柱)。在一些实施方案中,用具有在约 10-20mM(例如,约 10-18mM、10-16mM、10-15mM、12-20mM、14-18mM、14-16mM)的范围内的磷酸盐浓度的洗涤缓冲液洗涤上样的混合模式色谱柱(例如,HA 柱)。在一些实施方案中,用具有是或大于 10mM、11mM、12mM、13mM、14mM、15mM、16mM、17mM、18mM、19mM、20mM 的磷酸盐浓度的洗涤缓冲液洗涤上样的混合模式色谱柱(例如,HA 柱)。在一些实施方案中,用梯度磷酸盐缓冲液实现从混合模式色谱柱(例如,HA 柱)的洗脱。在一些实施方案中,适当的洗脱缓冲液可具有约 1-400mM(例如,1-300mM、1-200mM、1-150mM、1-100mM、10-350mM、10-300mM、10-250mM、10-200mM、10-150mM、10-140mM、10-130mM、10-120mM、10-110mM、10-100mM、10-90mM、10-80mM、10-70mM、10-60mM、10-50mM)的磷酸钠或磷酸钾的磷酸盐梯度。在一些实施方案中,通过逐步增加洗脱缓冲液中的磷酸盐浓度实现从 HA 柱的洗脱。在一些实施方案中,逐步洗脱缓冲液可具有选自 10mM、20mM、30mM、40mM、50mM、60mM、70mM、80mM、90mM、100mM、110mM、120mM、130mM、140mM、150mM、200mM、250mM、300mM、350mM、400mM 的磷酸盐(例如,磷酸钠)浓度。在一些实施方案中,通过具有在约 50mM 至 150mM 的范围内的磷酸盐(例如,磷酸钠)浓度(例如,选自 50mM、60mM、70mM、80mM、90mM、100mM、110mM、120mM、130mM、140mM、150mM 及其组合的磷酸盐(例如,磷酸钠)浓度)的洗脱缓冲液实现从混合模式色谱柱(例如,HA 柱)的洗脱。

[0017] 在一些实施方案中,在上样至疏水相互作用色谱柱(例如,苯基柱)之前,将不纯制剂或中间洗脱液或过柱液调整至 pH 为约 4.5-6.0(例如,约 4.5、5.0、5.5 或 6.0)的在 0.5M 至约 2.0M(例如,约 0.5M、1.0M、1.1M、1.2M、1.3M、1.4M、1.5M、1.6M、1.7M、1.8M、1.9M 或 2.0M NaCl)的范围内的盐(例如,NaCl)浓度。在一些实施方案中,上样后,使用 pH 为约 4.5-6.0(例如,约 4.5、5.0、5.5 或 6.0)的包含在约 0.5M 至 2.0M(例如,约 0.5M、1.0M、1.1M、1.2M、1.3M、1.4M、1.5M、1.6M、1.7M、1.8M、1.9M 或 2.0M NaCl)的范围内的盐(例如,NaCl)浓度的洗涤缓冲液洗涤疏水相互作用色谱柱。在一些实施方案中,使用 pH 为约 4.5-6.0(例如,约 4.5、5.0、5.5 或 6.0)的包含在约 0.1M 至约 0.5M(例如,约 0.1M、0.2M、0.3M、0.4M 或 0.5M NaCl)的范围内的盐(例如,NaCl)浓度的洗脱缓冲液洗脱疏水相互作用色谱柱。

[0018] 在一些实施方案中,阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱柱中的每一种具有在 14-25cm(例如,15-25cm、15-20cm、14-24cm、14-22cm、14-20cm 或 16-18cm)的范围内的高度。在一些实施方案中,阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱柱中的每一种具有约 14、15、16、17、18、19、20、21、22、23、24 或 25cm 的高度。

[0019] 在一些实施方案中,根据本发明的发明方法包括在将不纯制剂上样至第一色谱柱之前进行病毒灭活的步骤。在一些实施方案中,病毒灭活的步骤包括将去垢剂添加至不纯制剂。在一些实施方案中,根据本发明的发明方法还包括在最后一个色谱柱之后除去病毒的步骤。在一些实施方案中,本发明的方法还包括超滤和 / 或渗滤的步骤。在一些实施方

案中，超滤和／或渗滤的步骤包括将纯化的重组 I2S 蛋白交换至药物配制缓冲液中。

[0020] 在一些实施方案中，本发明用于纯化具有与 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，本发明用于纯化具有与 SEQ ID NO:1 相同的氨基酸序列的重组 I2S 蛋白。

[0021] 在一些实施方案中，本发明用于纯化由在无血清培养基中悬浮培养的哺乳动物细胞产生的重组 I2S 蛋白。在一些实施方案中，适合于本发明的无血清培养基缺乏动物来源的组分。在一些实施方案中，适合于本发明的无血清培养基是化学成分确定的培养基。在一些实施方案中，在生物反应器中培养哺乳动物细胞。在一些实施方案中，哺乳动物细胞共表达重组 I2S 蛋白和甲酰甘氨酸生成酶 (FGE)。在一些实施方案中，哺乳动物细胞为人细胞。

[0022] 在一些实施方案中，从含有由哺乳动物细胞分泌的重组 I2S 蛋白的无血清培养基制备用于本发明的方法的不纯制剂。在一些实施方案中，将用于本发明的方法的不纯制剂从冷冻的培养基制剂解冻。

[0023] 在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白含有平均每分子 16-22 个（例如，16-21 个、16-20 个、16-19 个、17-22 个、17-21 个、17-20 个、17-19 个）唾液酸。在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白含有平均每分子 16、17、18、19、20、21 或 22 个唾液酸。

[0024] 在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白具有至少约 70％（例如，至少约 77％、80％、85％、90％、95％、96％、97％、98％、99％）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，根据本发明的纯化的重组 I2S 蛋白具有大体上 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，根据本发明的纯化的重组 I2S 蛋白具有至少 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。

[0025] 在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白特征在于大于 70％、75％、80％、85％、90％、95％的细胞摄取，如通过体外摄取测定所测定的。

[0026] 在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白特征在于包含 7 个峰组的聚糖图谱，所述峰组分别指示中性（峰组 1）、单唾液酸化（峰组 2）、二唾液酸化（峰组 3）、单磷酸化（峰组 4）、三唾液酸化（峰组 5）、四唾液酸化（峰组 6）和二磷酸化（峰组 7）I2S 蛋白。在一些实施方案中，在神经氨酸酶消化后产生聚糖图谱。在其它实施方案中，在碱性磷酸酶消化后产生聚糖图谱。

[0027] 除其它事项以外，本发明提供了如本文所述的纯化的重组 I2S 蛋白以及包含所述重组 I2S 蛋白的药物组合物或制剂。在一些实施方案中，配制用于静脉内、皮下和／或鞘内施用的制剂。本发明还提供了通过向需要治疗的受试者施用纯化的重组 I2S、包含重组 I2S 的药物组合物或制剂来治疗亨特综合症的方法。

[0028] 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。

[0029] 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涵盖由相关领域的普通技术人员所理解的任何正常波动。

[0030] 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。

[0031] 附图简述

[0032] 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。

[0033] 图1描述了无血清培养基中产生的重组I2S的示例性纯化方案。

[0034] 图2描述了与参考I2S相比较的纯化的重组I2S AF的示例性肽图谱。

[0035] 图3描述了纯化的重组I2S AF的示例性SDS-PAGE（银）分析。

[0036] 图4描述了通过离子交换色谱评估的纯化的重组I2S AF的示例性电荷特征谱分析。

[0037] 图5描述了纯化的重组I2S AF的示例性聚糖特征图谱。

[0038] 图6描述了在病毒灭活UPB步骤后重组I2S的澄清收获物的活性(U/mg)的示例性分析。

[0039] 图7描述了在病毒灭活UPB步骤后重组I2S的澄清收获物的SE C-HPLC示例性分析。

[0040] 图8描述了纯化的重组I2S蛋白的利用银染处理的示例性SDS-P AGE。

[0041] 图9显示相较于参考，从在无血清培养条件（顶图）下生长的I2S-AF 2D细胞系产生的纯化的重组I2S酶的示例性肽图谱。

[0042] 图10描述了相较于参考，针对使用在无血清细胞培养条件下生长的I2S-AF 2D和4D细胞系产生的纯化的重组I2S酶产生的示例性聚糖特征谱。

[0043] 图11描述了相较于I2S参考对照，针对使用在无血清细胞培养条件下生长的I2S-AF 2D细胞系产生的纯化的重组I2S酶产生的示例性电荷征谱。

[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] 大致或约：如本文中所用，术语“大致”或“约”，如用于一个或多个目标值，意指与所述参考值相似的值。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）上落在所述参考值的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。

[0047] 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。

[0048] 不依赖于阳离子的甘露糖-6-磷酸受体(CI-MPR)：如本文中所用，术语“不依赖于阳离子的甘露糖-6-磷酸受体(CI-MPR)”是指结合注定被转运至溶酶体的高尔基体中的酸性水解酶前体上的甘露糖-6-磷酸(M6P)标签的细胞受体。除了甘露糖-6-磷酸以外，CI-MPR还结合其它蛋白质，包括IGF-II。CI-MPR也被称为“M6P/IGF-II受体”、“CI-MPR/IGF-II受体”、“IGF-II受体”或“IGF2受体”。这些术语及其缩写在本文可互换使用。

[0049] 色谱：如本文中所用，“色谱”是指用于混合物的分离的技术。通常情况下，将混合物溶解在称为“流动相”的流体中，所述流体携带其通过称为“固定相”的保持另一种材料的结构。柱色谱是其中固定床在管（即柱）内的分离技术。

[0050] 稀释剂：如本文中所用，术语“稀释剂”是指用于复水制剂的制备的药学上可接受的（例如，对于向人施用是安全和无毒的）稀释物质。示例性稀释剂包括无菌水、注射用抑菌水(BWFI)、pH缓冲液（例如，磷酸盐缓冲盐水）、无菌盐溶液、林格氏溶液(Ringer's solution)或葡萄糖溶液。

[0051] 洗脱：如本文中所用，术语“洗脱”是指通过用溶剂洗涤来从另一种材料提取一种材料的过程。例如，在离子交换色谱中，洗脱是洗涤上样树脂以除去捕获的离子的过程。

[0052] 洗脱液：如本文中所用，术语“洗脱液”是指通常作为洗脱结果的流动相“载体”与出自色谱的分析材料的组合。

[0053] 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。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。

[0054] 使平衡或平衡：如本文中所用，关于色谱的术语“使平衡”或“平衡”是指用另一种液体使第一液体（例如，缓冲液）平衡，通常以实现液体（例如，缓冲液）的组分的稳定和均衡分布的过程。例如，在一些实施方案中，色谱柱可通过使一个或或多个柱体积的所需液体（例如，缓冲液）通过柱来进行平衡。

[0055] 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）。

[0056] 杂质：如本文中所用，术语“杂质”是指有限量的液体、气体或固体内的与目标材料或化合物的化学组成不同的物质。杂质也称为污染物。

[0057] 接头：如本文中所用，“接头”是指在融合蛋白中，除在天然蛋白质中的特定位置上出现的氨基酸序列外的氨基酸序列，并且其通常被设计成柔性的或插入在两个蛋白质部分之间的结构，例如α-螺旋。接头也称为间隔子。

[0058] 上样：如本文中所用，术语“上样”是指在色谱中，将含有样品的液体或固体添加

至柱。在一些实施方案中，当上样的样品通过柱时，上样至柱上的样品的特定组分随后被捕获。在一些实施方案中，当上样的样品通过柱时，上样至柱上的样品的特定组分未被柱捕获或“流过”柱。

[0059] 多肽：如本文中所用，“多肽”，一般来说，是通过肽键彼此连接的至少两个氨基酸的串。在一些实施方案中，多肽可以包括至少3-5个氨基酸，其每一个通过至少一个肽键连接于其它氨基酸。本领域普通技术人员将理解，多肽有时任选地包括“非天然”氨基酸或然而能够任选地掺入多肽链的其它实体。

[0060] 汇集：如本文中所用，与色谱相关的术语“汇集”是指将一个或多个已通过柱的液体级分组合在一起。例如，在一些实施方案中，可将一个或多个已通过色谱分离的包含样品的期望组分的级分（例如，“峰级分”）“汇集”一起，产生单个“汇集”级分。

[0061] 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。

[0062] 可溶的：如本文中所用，术语“可溶的”是指治疗剂形成均质的溶液的能力。在一些实施方案中，治疗剂在其中被施用和借助其被转运至靶作用位点的溶液中的溶解度足以允许治疗有效量的治疗剂至靶作用位点的递送。若干因素可影响治疗剂的溶解度。例如，可影响蛋白质溶解度的相关因素包括离子强度、氨基酸序列和其它共增溶剂或盐（例如，钙盐）的存在。在一些实施方案中，根据本发明的治疗剂可溶于其相应的药物组合物。

[0063] 稳定性：如本文中所用的，术语“稳定的”是指治疗剂（例如，重组酶）在长时期内保持其治疗效能（例如，全部或大部分其预期的生物学活性和/或生理化学完整性）的能力。可在长时期内（例如，至少1，3，6，12，18，24，30、36个月或更长时间）评估治疗剂的稳定性和药物组合物维持这样的治疗剂的稳定性的能力。在制剂的情形中，稳定的制剂是如下的制剂，其中的治疗剂在贮存后和在处理（例如冷冻/解冻、机械混合和冷冻干燥）过程中基本上维持其物理和/或化学完整性和生物活性。对于蛋白质稳定性，其也可通过高分子量(HMW)聚集物的形成、酶活性的丧失、肽片段的产生和电荷特征谱的移位来测量。

[0064] 病毒处理：如本文中所用，术语“病毒处理”是指其中病毒被简单地从样品除去的“病毒去除”，或其中病毒保留在样品中但以非感染性形式存在的“病毒灭活”。在一些实施方案中，病毒去除可以利用除其它以外，纳米过滤和/或色谱技术。在一些实施方案中，病毒灭活可利用除其它以外，溶剂灭活、去垢剂灭活、巴氏消毒法、酸性pH灭活和/或紫外线灭活。

[0065] 发明详述

[0066] 本发明除其它事项以外提供了基于包括少于6个色谱步骤的方法的用于纯化用于酶替代疗法的重组I2S蛋白的改进的方法。在一些实施方案中，本发明提供了使用基于阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱中的一种或多种的工艺从不纯制剂种纯化重组I2S蛋白的方法。在一些实施方案中，本发明提供了通过进行

Q 色谱、羟基磷灰石（HA）色谱、SP 色谱、苯基色谱来从不纯制剂纯化重组 I2S 蛋白的方法。本发明还提供了纯化的重组 I2S 蛋白及使用方法。

[0067] 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”。

[0068] 重组 I2S 蛋白

[0069] 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。

[0070] 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。

[0071] 表 1. 人艾杜糖 -2- 硫酸酯酶

[0072]

成熟形式	SETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP(SEQ ID NO:1)
全长前体 (同种型a)	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPEAGEKLFPYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP(SEQ ID NO:2)
同种型b前体	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQEDQSSTGFRLKTSSTRKYK (SEQ ID NO:3)
同种型c前体	MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGFLMRTNT(SEQ ID No:4)

[0073] 因此,在一些实施方案中,重组 I2S 蛋白为成熟人 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,重组 I2S 蛋白可以是成熟人 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,重组 I2S 蛋白与成熟人 I2S 蛋白 (SEQ ID NO:1) 大体上同源。在一些实施方案中,重组 I2S 蛋白具有与 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,重组 I2S 蛋白与成熟人 I2S 蛋白 (SEQ ID NO:1) 大体上同一。在一些实施方案中,重组 I2S 蛋白具有与 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,重组 I2S 蛋白包含成熟人 I2S 蛋白的片段或部分。

[0074] 或者,重组 I2S 蛋白是全长 I2S 蛋白。在一些实施方案中,重组 I2S 蛋白可以是全长人 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,重组I2S蛋白与全长人I2S蛋白(SEQ ID NO:2)大体上同源。例如,重组I2S蛋白可具有与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,重组I2S蛋白与SEQ ID NO:2大体上同一。例如,重组I2S蛋白可具有与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,重组I2S蛋白包含全长人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。

[0075] 在一些实施方案中,重组I2S蛋白为人I2S同种型a蛋白。在一些实施方案中,重组I2S蛋白可以是人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,重组I2S蛋白与人I2S同种型a蛋白(SEQ ID NO:3)大体上同源。例如,重组I2S蛋白可具有与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,重组I2S蛋白与SEQ ID NO:3大体上同一。例如,重组I2S蛋白可具有与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,重组I2S蛋白包含人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。

[0076] 在一些实施方案中,重组I2S蛋白为人I2S同种型b蛋白。在一些实施方案中,重组I2S蛋白可以是人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,重组I2S蛋白与人I2S同种型b蛋白(SEQ ID NO:4)大体上同源。例如,重组I2S蛋白可具有与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,重组I2S蛋白与SEQ ID NO:4大体上同一。例如,重组I2S蛋白可具有与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,重组I2S蛋白包含人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。

[0077] 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。

[0078] 在一些实施方案中,重组I2S蛋白可包含结合于靶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。在一些实施方案中，重组I2S蛋白在蛋白质表面上包含M6P残基。具体地，重组I2S蛋白可包含具有更高的针对CI-MPR的结合亲和力的二磷酸化寡糖。在一些实施方案中，适合的酶包含每种酶高达约平均约至少20％的二磷酸化寡糖。在其它实施方案中，适合的酶可包含每种酶约10％、15％、18％、20％、25％、30％、35％、40％、45％、50％、55％、60％的二磷酸化寡糖。二磷酸化寡糖

[0079] 在一些实施方案中，可将重组I2S酶融合于能够结合靶细胞表面上的受体的溶酶体靶向部分。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）。可将溶酶体靶向部分在N末端、C末端或内部缀合或融合于I2S蛋白或酶。

[0080] 重组I2S蛋白的产生

[0081] 本发明可用于纯化通过各种方法产生的重组I2S蛋白。例如，可通过利用经工程化以表达I2S编码核酸的宿主细胞系统重组产生I2S蛋白。或者，可通过激活内源性I2S基因产生I2S蛋白。

[0082] 预期本发明可用于纯化使用各种表达系统产生的重组I2S蛋白。适当的表达系统包括例如蛋、杆状病毒、植物、酵母或哺乳动物细胞。

[0083] 在一些实施方案中，在哺乳动物细胞中产生I2S酶。可按照本发明使用的哺乳动物细胞的非限定性实例包括BALB/c小鼠骨髓瘤细胞系（NSO/1，ECACC No: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）；人胚肾系（被亚克隆来用于在悬浮培养中生长的HEK293或293细胞，Graham等人，J.Gen Virol.，36:59，1977）；人纤维肉瘤细胞系（例如，HT1080）；幼仓鼠肾细胞（BHK21，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（TM4，Mather，Biol.Reprod.，23:243-251，1980）；猴肾细胞（CV1ATCC CCL 70）；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 CCL 34）；buffalo大鼠肝细胞（BRL3A，ATCC CRL 1442）；人肺细胞（W138，ATCC CCL 75）；人肝细胞（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）；MRC5细胞；FS4细胞；和人肝细胞瘤细胞系（Hep G2）。

[0084] 在一些实施方案中，根据本发明的发明方法用于纯化从人细胞（例如，HT1080）产生的重组I2S酶。在一些实施方案中，根据本发明的发明方法用于纯化从CHO细胞产生的重组I2S酶。

[0085] 通常情况下，经工程化以表达重组I2S的细胞可包含编码本文中描述的重组I2S蛋白的转基因。应当理解，编码重组I2S的核酸可包含调控序列、基因控制序列、启动子、非编码序列和/或用于表达重组I2S的其它适当的序列。通常情况下，将编码区与这些核酸组件的一个或多个可操作地连接。

[0086] “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、翻译前导序列、内含子和多腺苷酸化识别序列。有时，“调控序列”也称为“基因控制序列”。

[0087] “启动子”通常是指能够控制编码序列或功能性 RNA 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不同的环境条件来指导基因的表达。

[0088] “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。

[0089] “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。

[0090] 通常情况下，术语“有效连接的”或“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。

[0091] 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。

[0092] 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。

[0093] 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。

[0094] 重组 I2S 蛋白的激活

[0095] 通常情况下，通过保守半胱氨酸（对应于成熟人 I2S 的氨基酸 59) 至甲酰甘氨酸（也称为 2- 氨基 -3- 氧代丙酸或氧代 - 丙氨酸）的翻译后修饰来激活重组 I2S 酶。这样的翻译后修饰可通过称为甲酰甘氨酸生成酶 (FGE) 的酶来进行。因此，在一些实施方案中，在也表达 FGE 蛋白的细胞中产生重组 I2S 酶。在具体实施方案中，在具有增加的或增强的 FGE 蛋白的表达的细胞中产生重组 I2S 酶。例如，细胞可经工程化以过表达与重组 I2S 组合的 FGE，以有利于具有高水平的活性酶的 I2S 制剂的产生。在一些实施方案中，通过使用标准重组技术表达（例如，过表达）外源 FGE 来实现 FGE 的过表达。在一些实施方案中，通过例

如激活或增强内源FGE基因的启动子激活或增强内源FGE的表达来实现FGE的过表达。在一些情况下，通过具有对应于内部核糖体进入位点的序列的核酸（例如，间隔子序列）来连接编码重组I2S的核酸与编码重组FGE蛋白的核酸。

[0096] 具有将半胱氨酸转化成甲酰甘氨酸的能力的任何FGE可用于本发明。用于FGE蛋白的示例性核酸和氨基酸序列公开于US2004-0229250，将与这样的序列相关的整个内容和序列本身通过引用整体并入本文中。应当理解，编码重组FGE的核酸可包含调控序列、基因控制序列、启动子、非编码序列和/或用于表达FGE的其它适当的序列。通常情况下，将编码区与这些核酸组件的一个或多个可操作地连接。

[0097] 细胞培养基和条件

[0098] 各种细胞培养基和条件可用于产生重组I2S蛋白。例如，可在含血清或无血清的培养基中产生重组I2S蛋白。在一些实施方案中，在无血清培养基中产生重组I2S蛋白。在一些实施方案中，在无动物的培养基，即缺乏动物来源组分的培养基中产生重组I2S蛋白。在一些实施方案中，在化学成分确定的培养基中产生重组I2S蛋白。如本文中所用，术语“化学成分确定的营养培养基”是指基本上所有的化学成分是已知的培养基。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如血清、血清来源的蛋白质（例如，白蛋白或胎球蛋白），以及其他组分。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，蛋白质生长因子或细胞因子）。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水解产物。在其他情况下，化学成分确定的营养培养基是不含蛋白质的培养基，即，不包含蛋白质、水解产物或未知组成的组分的无血清培养基。

[0099] 在一些实施方案中，化学成分确定的培养基可补充一种或多种动物来源组分。这样的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）。

[0100] 各种细胞培养条件可以用于大规模产生重组I2S蛋白，包括但不限于滚瓶培养、生物反应器分批培养和生物反应器补料分批培养。在一些实施方案中，重组I2S蛋白是由悬浮培养的细胞产生的。在一些实施方案中，重组I2S蛋白是通过贴壁细胞产生的。

[0101] 示例性细胞培养基和培养条件在实施例部分中进行了描述。用于产生重组I2S蛋白的另外的示例性方法和组合物在与其同一日提交的标题为“用于产生重组艾杜糖-2-硫酸酯酶的方法和组合物(Methods and Compositions for Producing Recombinant Iduronate-2-Sulfatase)”的临时申请中进行了描述，所述临时申请的全部公开内容通过引用并入本文。

[0102] 重组I2S蛋白的纯化

[0103] 在一些实施方案中，本发明提供了使用基于阴离子交换色谱、阳离子交换色谱、混合模式色谱和疏水相互作用色谱中的一种或多种的工艺从不纯制剂纯化重组I2S蛋白的方法。在一些实施方案中，根据本发明的发明方法包括少于6个（例如，少于5个、少于4个或少于3个）色谱步骤。在一些实施方案中，根据本发明的发明方法包括2、3、4或5个色谱步骤。在一些实施方案中，根据本发明的发明方法包括4个色谱步骤。在一些实施方案中，根据本发明的发明方法按该顺序包括阴离子交换色谱、混合模式色谱、阳离子交换色谱和疏水相互作用色谱。

[0104] 不纯制剂

[0105] 如本文中所用，不纯制剂可以是任何生物材料，包括含有重组 I2S 蛋白的未处理的生物材料。例如，不纯制剂可以是含有从产生 I2S 蛋白的细胞（例如，哺乳动物细胞）分泌的重组 I2S 蛋白的未处理的细胞培养基或含有 I2S 蛋白的原始细胞裂解物。在一些实施方案中，不纯制剂可以是部分处理的细胞培养基或细胞裂解物。例如，可浓缩、稀释、通过病毒灭活、病毒处理或病毒去除处理细胞培养基或细胞裂解物。在一些实施方案中，病毒去除可利用除其它以外的纳米过滤和／或色谱技术。在一些实施方案中，病毒灭活可利用除其它以外的溶剂灭活、去垢剂灭活、巴氏消毒法、酸性 pH 灭活和／或紫外灭活。还可利用蛋白酶、DNA 酶和／或 RNA 酶处理细胞培养基或细胞裂解物以降低宿主细胞蛋白质和／或核酸（例如，DNA 或 RNA）的水平。在一些实施方案中，可冷冻未处理的或部分处理的生物材料（例如，细胞培养基或细胞裂解物），并将其于期望的温度（例如，2-8℃、-4℃、-25℃、-75℃）下贮存一段时间，随后解冻用于纯化。如本文中所用，不纯制剂也称为起始材料或填料。

[0106] 阴离子交换色谱

[0107] 在一些实施方案中，提供的用于纯化重组 I2S 的方法包括阴离子交换色谱。简言之，阴离子交换色谱是依赖于带负电荷的化合物与带正电荷的树脂之间的电荷－电荷相互作用的色谱技术。在一些实施方案中，阴离子交换色谱为强阴离子交换色谱。在一些实施方案中，将阴离子交换色谱用作治疗性蛋白（例如，重组 I2S）的第一纯化步骤。

[0108] 示例性阴离子交换树脂包括但不限于季胺树脂或“Q－树脂”（例如，Capto™-Q、Q-Sepharose®、QAE Sephadex®）；二乙基氨基乙烷（DEAE）树脂（例如，DEAE-Trisacryl®、DEAE Sepharose®、苯甲酰化萘酰化 DEAE、二乙基氨基乙基 Sephacel®）；Amberjet®树脂；Amberlyst®树脂；Amberlite®树脂（例如，Amberlite® IRA－67、Amberlite® 强碱性、Amberlite®弱碱性）、考来烯胺树脂、ProPac®树脂（例如，ProPac® SAX－10、ProPac® WAX－10、ProPac® WCX－10）；TSK-GEL®树脂（例如，TSKgel DEAE－NPR；TSKgel DEAE－5PW）；和 Acclaim®树脂。在某些实施方案中，阴离子交换树脂为 Q 树脂。

[0109] 用于阴离子交换色谱的典型流动相包括相对极性溶液，例如水、乙腈、有机醇例如甲醇、乙醇和异丙醇或包含 2-(N- 吗啉)- 乙磺酸 (MES) 的溶液。因此，在某些实施方案中，流动相包含约 0％、1％、2％、4％、6％、8％、10％、12％、14％、16％、18％、20％、25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％或约 100％的极性溶液。在某些实施方案中，流动相在分离过程中的任意给定的时间包含约 1％至约 100％、约 5％至约 95％、约 10％至约 90％、约 20％至约 80％、约 30％至约 70％或约 40％至约 60％的极性溶液。

[0110] 一般地，流动相包含盐。例如，盐（例如，氯化钠）可从阴离子交换柱（例如，抗衡离子为氯离子并且其用于与靶蛋白质交换，随后所述靶蛋白质被释放）洗脱结合的蛋白质。在一些实施方案中，流动相包含约 0 至约 1.0M，例如约 0 至约 0.8M、约 0 至约 0.6M、约 0 至约 0.5M、约 0 至约 0.4M、约 0.05M 至约 0.50M、约 0.10M 至约 0.45M、约 0.10M 至约 0.40M 或约 0.15M 至约 0.40M 的盐浓度。在一些实施方案中，流动相包含大致 0.01M、0.02M、0.03M、 0.04M、0.05M、0.06M、0.07M、0.08M、0.09M、0.1M、0.2M、0.3M、0.4M、0.5M、0.6M、0.7M、0.8M、0.9M或1.0M的盐浓度。在一些实施方案中，流动相中的盐浓度是梯度（例如，线性或非线性的梯度）。在一些实施方案中，流动相中的盐浓度是恒定的。在一些实施方案中，流动相中的盐浓度可逐步增加或减小。

[0111] 通常情况下，对流动相进行缓冲。在某些实施方案中，不对流动相进行缓冲。在某些实施方案中，将流动相缓冲至约5至约14的pH。在某些实施方案中，将流动相缓冲至约5至约10的pH。在某些实施方案中，将流动相缓冲至约5至约7的pH。在某些实施方案中，将流动相缓冲至约6.5的pH。在某些实施方案中，将流动相缓冲至约5.0、5.5、6.0、6.5、7.0、7.5、8.0、8.5、9.0、9.5或10的pH。

[0112] 在一些实施方案中，在上样至阴离子交换色谱柱（例如，Q柱）之前，将不纯制剂或中间洗脱液或过柱液调整至约5.0、5.5、6.0、6.5、7.0或7.5的pH和约2mS/cm、4mS/cm、6mS/cm、8mS/cm、10mS/cm、12mS/cm、14mS/cm、16mS/cm、18mS/cm或20mS/cm的电导率。可使用醋酸钠（例如，1M）调整pH，以及可使用氯化钠（例如，5M）调整电导率。上样后，可使用pH为约5.0-7.5(例如，约5.0、5.5、6.0、6.5、7.0或7.5)的包含在约140mM至约200mM(例如，约140mM、145mM、150mM、155mM、160mM、165mM、170mM、175mM、180mM、185mM、190mM、195mM或200mM)的范围内的盐(例如，NaCl)浓度的洗涤缓冲液洗涤阴离子交换色谱柱。可使用包含线性NaCl梯度的洗脱缓冲液洗脱阴离子交换色谱柱。适合的示例性线性NaCl梯度可包含约0-500mM NaCl(例如，约0-400mM、约0-350mM、约0-300mM、约50-500mM、约150-500mM、约150-450mM、约150-400mM)的范围。

[0113] 阳离子交换色谱

[0114] 在一些实施方案中，提供的用于纯化重组I2S的方法包括阳离子交换色谱。简言之，阳离子交换色谱是依赖于带正电荷的化合物与带负电荷的树脂之间的电荷－电荷相互作用的色谱技术。在一些实施方案中，阳离子交换色谱是强阳离子交换色谱。

[0115] 通常用强或弱阳离子交换柱实施阳离子交换色谱，所述色谱柱包含锍离子或具有弱阳离子交换剂（通常具有羧甲基(CM)或羧化物(CX)官能团）。许多适合的阳离子交换树脂在本领域是已知的，并且是商购可得的，并且包括但不限于SP-Sepharose®、CM Sepharose®；Amberjet®树脂；Amberlyst®树脂；Amberlite®树脂（例如，Amberlite® IRA120)；ProPac®树脂（例如，ProPac® ScX－10、ProPac® WCX－10、ProPac® WCX－10)；TSK-GEL®树脂（例如，TSKgel BioAssist S；TSKgel SP-2SW、TSKgel SP－5PW；TSKgel SP-NPR；TSKgel SCX；TSKgel SP-STAT；TSKgel CM-5PW；TSKgel OApak-A；TSKgel CM-2SW、TSKgel CM-3SW和TSKgel CM-STAT)；以及Acclaim®树脂。在某些实施方案中，阴离子交换树脂为SP-Sepharose resin®。

[0116] 用于阳离子交换色谱的典型流动相包含相对极性的溶液，例如水、乙腈、有机醇例如甲醇、乙醇和异丙醇或包含2-(N-吗啉)-乙磺酸(MES)的溶液。因此，在某些实施方案中，流动相包含约0％、1％、2％、4％、6％、8％、10％、12％、14％、16％、18％、20％、25％、30％、35％、40％、45％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％或约100％的极性溶液。在某些实施方案中，流动相在分离过程中的任意给定的时间包含约1％

至约 100％、约 5％至约 95％、约 10％至约 90％、约 20％至约 80％、约 30％至约 70％或约 40％至约 60％的极性溶液。

[0117]　通常地，流动相包含盐。例如，盐（例如，氯化钠、磷酸钠等）可从阳离子交换柱（例如，抗衡离子为钠离子，并且其与靶蛋白质交换，随后所述蛋白质被释放）洗脱结合的蛋白质。在一些实施方案中，流动相包含约 0 至约 1.0M，例如约 0 至 0.8M、约 0 至约 0.6M、约 0 至约 0.5M、约 0 至约 0.4M、约 0.05M 至约 0.50M、约 0.10M 至约 0.45M、约 0.10M 至约 0.40M 或约 0.15M 至约 0.40M 的盐浓度。在一些实施方案中，流动相包含大致 0.01M、0.02M、0.03M、0.04M、0.05M、0.06M、0.07M、0.08M、0.09M、0.1M、0.2M、0.3M、0.4M、0.5M、0.6M、0.7M、0.8M、0.9M 或 1.0M 的盐浓度。在一些实施方案中，流动相中的盐浓度是梯度（例如，线性或非线性的梯度）。在一些实施方案中，流动相中的盐浓度是恒定的。在一些实施方案中，流动相中的盐浓度可逐步增加或减小。

[0118]　通常情况下，对流动相进行缓冲。在某些实施方案中，不对流动相进行缓冲。在某些实施方案中，将流动相缓冲至约 5 至约 14 的 pH。在某些实施方案中，将流动相缓冲至约 5 至约 10 的 pH。在某些实施方案中，将流动相缓冲至约 5 至约 7 的 pH。在某些实施方案中，将流动相缓冲至约 6.5 的 pH。在某些实施方案中，将流动相缓冲至约 5.0、5.5、6.0、6.5、7.0、7.5、8.0、8.5、9.0、9.5 或 10 的 pH。

[0119]　在一些实施方案中，在上样至阳离子交换色谱柱（例如，SP 柱）之前，将不纯制剂或中间洗脱液或过柱液调整至在约 1mS/cm 至 20mS/cm(例如，约 1mS/cm 至 15mS/cm、约 1mS/cm 至 10mS/cm、约 1mS/cm 至 8mS/cm、约 1mS/cm 至 6mS/cm、约 1mS/cm 至 4mS/cm、约 2mS/cm 至 4mS/cm) 的范围内的电导率。在具体实施方案中，在上样至阳离子交换色谱柱（例如，SP 柱）之前，将不纯制剂或中间洗脱液或过柱液调整至在约 2mS/cm 至 4mS/cm(例如，2、2.5、3、3.5 或 4mS/cm) 的范围内的电导率。可通过用 H_2O 以例如 1:1、1.1:1、1.2:1、1.3:1、1.4:1、1.5:1、2.0:1、2.5:1、3.0:1、4.0:1、5.0:1 或 10:1 的比率稀释不纯制剂或中间洗脱液或过柱液来调整电导率。还可通过渗滤至期望的缓冲液来调整电导率。在一些实施方案中，在约 5.0-6.5(例如，约 5.0、5.5、6.0 或 6.5) 的 pH 下运行阳离子交换色谱柱。在一些实施方案中，利用包含在约 0.01M 至约 0.1M(例如，约 0.01M、0.02M、0.03M、0.04M、0.05M、0.06M、0.07M、0.08M、0.09M 或 0.1M) 的范围内的磷酸盐（例如，NaPO4) 浓度的缓冲液运行阳离子交换色谱柱。在一些实施方案中，合适的 pH 为约 5.0-6.5(例如，约 5.0、5.5、6.0 或 6.5)。

[0120]　混合模式色谱

[0121]　羟基磷灰石色谱 (HA) 被认为是“假亲和”色谱或“混合模式”离子交换，并且可按照本发明使用。羟基磷灰石是用于生物分子的分级分离和纯化的磷酸钙的独特形式。在一些情况下，可使用结晶羟基磷灰石，尽管晶体的脆性可限制流速和／或柱寿命。两种类型的化学纯陶瓷羟基磷灰石，CHT 陶瓷羟基磷灰石 I 型和 II 型，是多孔的、球状的，并且可在高流速和压力下使用。I 型通常具有高蛋白质结合能力，而 II 型通常具有较低的对于蛋白质的结合能力。通常地，羟基磷灰石的分子式为 $Ca_{10}(PO_4)_6(OH)_2$(Kawasaki, 等人 1985)。官能团包括带正电荷的晶体钙离子对 (C- 位点）和 6 个与三个一组的晶体磷酸盐缔合的带负电荷的氧原子的簇 (P- 位点）。C- 位点、P- 位点和羟基以固定模式分布在晶体表面上，通常导致与蛋白质和其它分子的复杂相互作用。

[0122]　可将样品上样至中性或近中性 pH 的低离子强度磷酸盐缓冲液（例如，1-10mM 磷酸

钠或磷酸钾）中的HA柱上。在一些实施方案中，在上样至混合模式色谱柱（例如，HA柱）之前，将不纯制剂或中间洗脱液或过柱液调整至在约0.001M至约0.01M(例如，约0.001M、0.002M、0.003M、0.004M、0.005M、0.006M、0.007M、0.008M、0.009M或0.01M)的范围内的磷酸盐（例如，NaPO4)浓度和约5.0-6.5(例如，约5.0、5.5、6.0或6.5)的pH。通常用具有可与上样缓冲液的浓度相当的磷酸盐浓度的洗涤缓冲液洗涤上样的HA柱。在一些实施方案中，在上样后，用中性或近中性pH的包含磷酸盐（例如，1-10mM磷酸钠或磷酸钾）的洗涤缓冲液洗涤混合模式色谱柱（例如，HA柱）。例如，适合的洗涤缓冲液可具有约1mM、2mM、3mM、4mM、5mM、6mM、7mM、8mM、9mM或10mM的磷酸盐浓度。在一些实施方案中，可期望增加洗涤缓冲液中的磷酸盐的量以产生更严格的洗涤条件。预期I2S蛋白表面上的M6P水平，具体为二-M6P水平对于溶酶体靶向是重要的。洗涤缓冲液中增加的磷酸盐浓度可选择性地将具有高水M6P具体为二-M6P的I2S蛋白保留在HA柱上。因此，在一些实施方案中，期望的洗涤缓冲液可具有为或大于10mM、11mM、12mM、13mM、14mM、15mM、16mM、17mM、18mM、19mM、20mM的磷酸盐浓度。在一些实施方案中，用具有在约10-20mM(例如，约10-18mM、10-16mM、10-15mM、12-20mM、14-18mM、14-16mM)的范围内的磷酸盐浓度的洗涤缓冲液洗涤上样的混合模式色谱柱（例如，HA柱）。在一些实施方案中，利用具有为或大于10mM、11mM、12mM、13mM、14mM、15mM、16mM、17mM、18mM、19mM、20mM的磷酸盐浓度的洗涤缓冲液洗涤上样的混合模式色谱柱（例如，HA柱）。

[0123] 通常用梯度磷酸盐缓冲液实现从HA柱的洗脱。例如，适合的洗脱缓冲液可具有约1-400mM(例如，1-300mM、1-200mM、1-150mM、1-100mM、10-350mM、10-300mM、10-250mM、10-200mM、10-150mM、10-140mM、10-130mM、10-120mM、10-110mM、10-100mM、10-90mM、10-80mM、10-70mM、10-60mM、10-50mM)的磷酸钠的磷酸盐梯度。在一些实施方案中，通过逐步增加洗脱缓冲液中的磷酸盐浓度来实现从HA柱的洗脱。在一些实施方案中，逐步洗脱缓冲液可具有选自10mM、20mM、30mM、40mM、50mM、60mM、70mM、80mM、90mM、100mM、110mM、120mM、130mM、140mM、150mM、200mM、250mM、300mM、350mM、400mM的磷酸盐浓度。在一些实施方案中，通过具有在约50mM至约150mM的范围内的磷酸盐（例如，磷酸钠）浓度（例如，选自约50mM、60mM、70mM、80mM、90mM、100mM、110mM、120mM、130mM、140mM、150mM及其组合的磷酸盐（例如，磷酸钠）浓度））的洗脱缓冲液实现从混合模式色谱柱（例如，HA柱）的洗脱。

[0124] 应理解，用于HA色谱的条件的许多不同组合是已知的并且可用于调整参数以适合用于特定目标蛋白质（例如，重组I2S)。

[0125] 疏水相互作用色谱

[0126] 疏水相互作用色谱(HIC)是使用疏水性的性质将蛋白质彼此分离的分离技术。在该类型的色谱中，将疏水基团例如苯基、辛基或丁基连接于固定柱。通过在表面上具有疏水氨基酸侧链的柱的蛋白质能够与柱上的疏水基团相互作用或与其结合。HIC柱是已知的，并且包括例如苯基琼脂糖

[0127] HIC分离通常使用离子交换色谱中使用的那些条件的相反条件来设计。一般地，最初将具有高离子强度的缓冲液，通常为硫酸铵施加到柱。缓冲液中的盐减弱样品溶质的溶剂化作用，从而随着溶剂化减弱，开始暴露的疏水区域被介质吸附。固定相通常被设计来与其它分子形成疏水性相互作用。然而，这些相互作用在水中通常太弱，以致于向缓冲液中添加盐（例如，Na_2SO_4、K_2SO_4、$(NH_4)_2SO_4$、NaCl、NH_4Cl、NaBr和NaSCN)导致疏水性相互作用。在

一些实施方案中，流动相包含约 0.1M 至约 3.0M，例如，约 0.1M 至约 1.5M，约 0.2M 至约 0.8M 或约 0.3M 至约 0.5M 的盐浓度。

[0128] 在某些实施方案中，对流动相进行缓冲。在某些实施方案中，不对流动相进行缓冲。在某些实施方案中，将流动相缓冲至约 5 至约 14 的 pH。在某些实施方案中，将流动相缓冲至约 5 至约 10 的 pH。在某些实施方案中，将流动相缓冲至约 5 至约 7 的 pH。在某些实施方案中，将流动相缓冲至约 5.0 的 pH。

[0129] 在一些实施方案中，在上样至疏水相互作用色谱柱（例如，苯基柱）之前，将不纯制剂或中间洗脱液或过柱液调整至 pH 为约 4.5-6.0（例如，约 4.5、5.0、5.5 或 6.0）的在约 0.5M 至约 2.0M（例如，约 0.5M、1.0M、1.1M、1.2M、1.3M、1.4M、1.5M、1.6M、1.7M、1.8M、1.9M 或 2.0M）的范围内的盐（例如，NaCl）浓度。上样后，可使用 pH 为约 4.5-6.0（例如，约 4.5、5.0、5.5 或 6.0）的包含在约 0.5M 至约 2.0M（例如，约 0.5M、1.0M、1.1M、1.2M、1.3M、1.4M、1.5M、1.6M、1.7M、1.8M、1.9M 或 2.0M）的范围内的盐（例如，NaCl）浓度的洗涤缓冲液洗涤疏水相互作用色谱柱。在一些实施方案中，使用 pH 为约 4.5-6.0（例如，约 4.5、5.0、5.5 或 6.0）的包含在约 0.1M 至约 0.5M（例如，约 0.1M、0.2M、0.3M、0.4M 或 0.5M）的范围内的盐（例如，NaCl）浓度的洗脱缓冲液洗脱疏水相互作用色谱柱。

[0130] 纯化的 I2S 蛋白的表征

[0131] 可使用各种方法表征纯化的重组 I2S 蛋白。

[0132] 纯度

[0133] 纯化的重组 I2S 蛋白的纯度通常通过存在于终产物中的各种杂质（例如，宿主细胞蛋白或宿主细胞 DNA）的水平来测量。例如，宿主细胞蛋白（HCP）的水平可通过 ELISA 或 SDS-PAGE 来测量。在一些实施方案中，纯化的重组 I2S 蛋白包含少于 150ng HCP/mg 的 I2S 的蛋白（例如，少于 140、130、120、110、100、90、80、70、60、50、40、30、30、20、10ng HCP/mg 的 I2S 蛋白）。在一些实施方案中，纯化的重组 I2S 蛋白，当经历利用银染的 SDS-PAGE 时，不具有拥有大于 0.05％、0.01％、0.15％、0.2％、0.25％、0.3％、0.35％、0.4％、0.45％或 0.5％的测定对照的强度的新条带。可使用各种测定对照，特别是可被管理机构例如 FDA 接受的那些测定对照。

[0134] 比活性

[0135] 纯化的重组 I2S 蛋白还可通过评价功能和/或生物活性来表征。重组 I2S 组合物的酶活性可使用本领域已知的方法来测定。通常情况下，该方法包括检测硫酸酯从合成底物的去除，这称为硫酸酯释放测定。酶活性测定的一个实例牵涉离子色谱的使用。该方法定量通过重组 I2S 从底物酶促释放的硫酸根离子的量。底物可以是天然底物或合成底物。在一些情况下，底物为硫酸肝素（例如，肝素二糖）、硫酸皮肤素或其功能等同物。通常情况下，释放的硫酸根离子通过具有电导率检测器的离子色谱进行分析。在本实例中，结果表示为 U/mg 蛋白质，其中 1 个单位被定义为每小时从底物释放 1 微摩尔硫酸根离子所需的酶的量。在一些实施方案中，纯化的重组 I2S 蛋白具有在约 0-100U/mg、约 10-100U/mg、约 10-80U/mg、约 20-80U/mg、约 20-70U/mg、约 20-60U/mg、约 20-50U/mg、约 30-100U/mg、约 30-90U/mg、约 30-80U/mg、约 30-70U/mg、约 30-60U/mg、约 40-100U/mg、约 40-90U/mg、约 40-80U/mg、约 40-70U/mg、约 40-60U/mg 的范围内的比活性，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量的。在一些实施方案中，纯化的重组 I2S 蛋白具有至

少约 5U/mg、约 10U/mg、约 15U/mg、约 20U/mg、约 25U/mg、约 30U/mg、约 35U/mg、约 40U/mg、约 45U/mg、约 50U/mg、约 55U/mg、约 60U/mg、约 65U/mg、约 70U/mg、约 75U/mg、约 80U/mg、约 85U/mg、约 90U/mg、约 95U/mg 或约 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法测量的。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 (pH 6)，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 1.0mL/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。

[0136] 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 0.5U/mg、1.0U/mg、1.5U/mg、2U/mg、2.5U/mg、3U/mg、4U/mg、5U/mg、6U/mg、7U/mg、8U/mg、9U/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。随后可通过将酶活性除以蛋白质浓度来计算比活性。

[0137] 在任一实例中，重组 I2S 组合物的蛋白质浓度可通过本领域已知的用于测定蛋白质浓度的任何适合的方法来测定。在一些情况下，蛋白质浓度通过紫外线吸收测定来测定。这样的吸收测定法通常在约 280nm 的波长 (A_{280}) 下进行。

[0138] 电荷特征谱

[0139] 纯化的重组 I2S 可通过与蛋白质相关的电荷特征谱来表征。通常情况下，蛋白质电荷特征谱反映了通常存在于蛋白质表面上的残基侧链电荷的模式。电荷特征谱可通过对蛋白质进行离子交换 (IEX) 色谱（例如，HPLC）测定来测定。在一些实施方案中，“电荷特征谱”是指一组代表在向柱添加包含交换离子的流动相后的某个时间点上从离子交换柱洗脱的蛋白质的量。

[0140] 通常情况下，适合的离子交换柱为阴离子交换柱。例如，电荷特征谱可通过使用高效液相色谱（HPLC）系统的强阴离子交换（SAX）色谱来测定。一般而言，重组 I2S 吸附至强阴离子交换柱的固定的正电荷上，并以预定流速使用流动相的递增离子强度的梯度与它们与带正电荷的柱的离子相互作用的强度成比例地从柱洗脱重组 I2S 种类。带较多负电荷的（较酸性的）I2S 种类比带较少负电荷的（较不酸性的）I2S 种类较晚洗脱出来。洗脱物中蛋白质的浓度通过紫外线吸收（在 280nm）来检测。

[0141] 在一些实施方案中，重组 I2S 在约 pH 8.0 下于 20mM TRIS-HCl 中吸附至 Mini Q PE 柱的固定正电荷上，并以 0.8ml/ 分钟的流速使用由 20mM TRIS-HCL、1M 氯化钠，pH 8.0 组成的流动相的递增离子强度的梯度与它们与带正电荷的柱的离子相互作用的强度成比例地从柱洗脱重组 I2S 种类。

[0142] 在一些实施方案中，可通过从 HPLC 柱洗脱后吸收单位对时间的色谱图描述电荷特征谱。色谱图可包含一组一个或多个峰，其中组中的每一个峰鉴定组合物的具有相似表面电荷的重组 I2S 的亚群。

[0143] 在一些实施方案中，纯化的 I2S 蛋白组合物在其电荷特征谱中显示至少 6 个峰。I2S 的示例性电荷特征谱描述于实施例部分和图 11 中。如图 11 中显示的，按递增的负电荷和递减的对色谱图的总峰面积的贡献标记（A 至 F）6 个峰。在一些实施方案中，取决于蛋白质表面上负电荷或正电荷的量，纯化的重组 I2S 组合物的电荷特征谱包含不同数目、尺寸、形状或时间间隔的峰。在一些实施方案中，重组 I2S 组合物具有少于 6 个（例如，少于 5、4、3 或 2 个）峰的电荷特征谱。在一些实施方案中，重组 I2S 的电荷特征谱可具有 5、4、3、2 或 1 个峰。例如，峰 A、B、C、D、E 和 F 的任何 1、2、3、4 或 5 个峰可以不存在于纯化的重组 I2S 蛋白组合物中或在所述组合物中减少。通常情况下，如果存在更少的峰，则电荷特征谱被认为更均一。

[0144] 聚糖图谱

[0145] 在一些实施方案中，纯化的重组 I2S 蛋白的特征可在于它们的蛋白聚糖组成，通常地称为聚糖图谱。不希望受任何理论束缚，据认为聚糖连接与分支结构的形状和复杂度一起可影响体内清除、溶酶体靶向、生物利用度和 / 或效力。

[0146] 通常情况下，聚糖图谱可通过酶促消化和随后色谱分析来测定。各种酶可用于酶促消化，包括但不限于适合的糖基化酶、肽酶（例如，内肽酶、外肽酶）、蛋白酶和磷酸酶。在一些实施方案中，适合的酶为碱性磷酸酶。在一些实施方案中，适合的酶为神经氨酸酶。聚糖（例如，磷聚糖）可通过色谱谱分析来检测。例如，磷聚糖可通过高效阴离子交换色谱 - 脉冲安培检测（HPAE-PAD）或尺寸排阻高效液相色谱（HPLC）来检测。由聚糖图谱上的每一个峰代表的聚糖（磷聚糖）的量可按照本领域已知的和本文中公开的方法，使用聚糖（例如，磷聚糖）的标准曲线来计算。

[0147] 在一些实施方案中，根据本发明的纯化的 I2S 显示聚糖图谱，该图谱包含 7 个分别指示中性（峰组 1）、单唾液酸化（峰组 2）、二唾液酸化（峰组 3 组）、单磷酸化（峰组 4）、三唾液酸化（峰组 5）、四唾液酸化（峰组 6）和二磷酸化（峰组 7）I2S 蛋白的峰组。I2S 的示例性聚糖图谱描述于图 10 中。在一些实施方案中，纯化的重组 I2S 具有少于 7 个峰组的聚糖图谱（例如，具有 6、5、4、3 或 2 个峰组的聚糖图谱）。在一些实施方案中，纯化的重组 I2S 具有拥有超过 7 个峰组（例如，8、9、10、11、12 或更多个）的聚糖图谱。

[0148] 对应于每一个峰组的聚糖的相对量可基于相对于预定参考标准中的相应峰组面积的峰组面积来测定。在一些实施方案中，峰组 1（中性）可具有相对于参考标准中的对应峰组面积在约 40-120％（例如，约 40-115％、约 40-110％、约 40-100％、约 45-120％、约 45-115％、约 45-110％、约 45-105％、约 45-100％、约 50-120％、约 50-110％）的范围内的峰组面积。在一些实施方案中，峰组 2（单唾液酸化的）可具有相对于参考标准中的对应峰组面积在约 80-140％（例如，约 80-135％、约 80-130％、约 80-125％、约 90-140％、约 90-135％、约 90-130％、约 90-120％、约 100-140％）的范围内的峰组面积。在一些实施方案中，峰组 3（二唾液酸化）可具有相对于参考标准中的对应峰组面积在约 80-110％（例如，约 80-105％、为约 80-100％、约 85-105％、约 85-100％）的范围内的峰组面积。在一些实施方案中，峰组 4（单磷酸化）可具有相对于参考标准中的对应峰组面积在约 100-550％（例如，约 100-525％，约 100-500％，约 100-450％，约 150-550％，约 150-500％，约 150-450％，约 200-550％，约 200-500％，约 200-450％，约 250-550％，约 250-500％，约 250-450％或约 250-400％）的范围内的峰组面积。在一些实施方案中，峰组 5（三唾液酸化）可具有相对于参考标准中的对应峰组面积在约 70-110％（例如，约 70-105％，约 70-100％，约 70-95％，约 70-90％，约 80-110％，约 80-105％，约 80-100％或约 80-95％）的范围内的峰组面积。在一些实施方案中，峰组 6（四唾液酸化的）可具有相对于参考标准中的对应峰组面积在约 90-130％（例如，约 90-125％，约 90-120％，约 90-115％，约 90-110％，约 100-130％，约 100-125％或约 100-120％）的范围内的峰组面积。在一些实施方案中，峰组 7（二磷酸化）可具有相对于参考标准中的对应峰组面积在约 70-130％（例如，约 70-125％，约 70-120％，约 70-115％，约 70-110％，约 80-130％，约 80-125％，约 80-120％，约 80-115％，约 80-110％，约 90-130％，约 90-125％，约 90-120％、约 90-115％，约 90-110％）的范围内的峰组面积。聚糖图谱的各种参考标准在本领域是已知的并且可以用于实施本发明。通常情况下，峰组 7（二磷酸化）对应于纯化的重组 I2S 蛋白的表面上的二 M6P 的水平。

[0149] 预期纯化的 I2S 的糖基化模式影响溶酶体靶向。各种体外细胞摄取测定在本领域是已知的并且可以用来实施本发明。例如，为了评估 M6P 受体对 I2S 的摄取，使用在其表面上表达 M6P 受体的人成纤维细胞进行细胞摄取测定。I2S 的内化量可通过 ELISA 法进行测定。在一些实施方案中，根据本发明的纯化的重组 I2S 蛋白的特征在于大于 70％、75％、80％、85％、90％、95％的细胞摄取，如通过体外摄取测定所测定的。

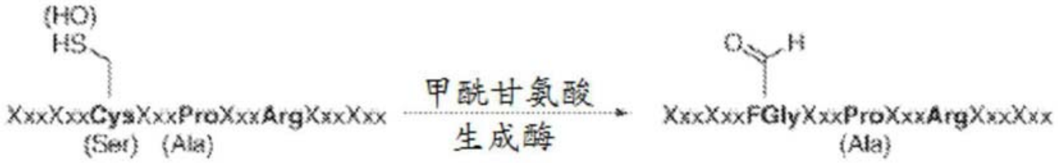
[0150] 肽图谱

[0151] 在一些实施方案中，肽图谱可用于表征氨基酸组成、翻译后修饰和／或细胞处理；例如信号肽的切割、甲酰甘氨酸转化和／或糖基化。通常情况下，重组蛋白可以被分解成离散的肽片段（通过受控或随机断裂），以产生模式或肽图谱。在一些情况下，可使纯化的 I2S 蛋白首先经历酶促消化，随后进行解析分析。消化可使用肽酶、糖苷水解酶、磷酸酶、脂肪酶或蛋白酶和／或其组合来进行，随后进行解析分析。肽的结构组成可使用本领域中公知的方法来确定。示例性的方法包括，但不限于，质谱、核磁共振（NMR）或 HPLC。

[0152] 甲酰甘氨酸转化百分比

[0153] 肽图谱分析可用于测定 FGly 转化百分比。如上所述讨论的，I2S 激活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，如下所示的：

[0154]



[0155] 因此，可使用下列公式计算甲酰甘氨酸转化的百分比（％ FG）：

[0156]

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

[0157] 为了计算％ FG，可使用蛋白酶（例如，胰蛋白酶或糜蛋白酶）将重组 I2S 蛋白消化成短肽。可使用例如尺寸排阻高效液相色谱（HPLC）分离和表征短肽。可表征包含对应于成熟人 I2S 的位置 59 的位置的肽以确定位置 59 上的 Cys 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）是否被转化成 FGly。包含 FGly 的蛋白质的量（对应于活性 I2S 分子的数目）以及具有 FGly 和 Cys 的肽的总量（对应于总的 I2S 分子的数目）可基于相应的峰面积来测定，并且可计算反映％ FG 的比率。

[0158] 在一些实施方案中，根据本发明的纯化的重组 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，根据本发明的纯化的重组 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。

[0159] 唾液酸含量

[0160] 在一些实施方案中，纯化的重组 I2S 蛋白的特征大于它们的唾液酸组成。不希望受理论的束缚，预期蛋白质上的唾液酸残基可通过存在于肝细胞上的无唾液酸糖蛋白受体阻止、减少或抑制其快速体内清除。因此，认为具有相对高的唾液酸含量的重组蛋白通常具有相对长的体内循环时间。

[0161] 在一些实施方案中，纯化的重组 I2S 蛋白的唾液酸含量可以使用本领域中公知的方法来确定。例如，重组蛋白的 I2S 的唾液酸含量可通过酶促消化和随后的色谱分析来确定。酶消化可以使用任何适合的唾液酸酶来完成。在一些情况下，消化是由糖苷水解酶进行的，如神经氨酸酶。唾液酸可通过色谱分析例如具有脉冲安培检测（HPAE-PAD）的高效阴离子交换色谱来检测。重组 I2S 组合物中的唾液酸的量可按照本领域中已知的和本文中公开的方法，使用唾液酸的标准曲线来计算。

[0162] 在一些实施方案中，纯化的重组 I2S 蛋白的唾液酸含量可以大于 16mol/mol。在唾液酸含量的上下文中，单位“mol/mol”是指每摩尔酶的唾液酸残基的摩尔数。在一些情况下，重组 I2S 蛋白的唾液酸含量为或大于约 16.5mol/mol、约 17mol/mol、约 18mol/mol、约 19mol/mol、约 20mol/mol、约 21mol/mol、约 22mol/mol 或更多。在一些实施方案中，纯化的重组 I2S 蛋白的唾液酸含量可以在约 16-20mol/mol、16-21mol/mol 之间、约 16-22mol/mol、16-23mol/mol、16-24mol/mol、约 16-25mol/mol、约 17-20mol/mol、17-21mol/mol、约 17-22mol/mol、17-23mol/mol、17-24mol/mol 或约 17-25mol/mol 的范围内。

[0163] 药物组合物和施用

[0164] 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。

[0165] 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。

[0166] 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。

[0167] 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。

[0168] 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。

[0169] 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。

[0170] 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。

[0171] 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。

[0172] 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。

[0173] 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。

[0174]　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。

[0175]　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。

[0176]　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。

[0177]　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

[0178]　实施例1：重组I2S AF捕获和纯化工艺

[0179]　本实施例说明简化的下游纯化工艺可以用于捕获和纯化在无血清培养基中产生的重组I2S。示例性纯化方案描述图1中。

[0180]　开发稳定地表达艾杜糖-2-硫酸酯酶（I2S）和甲酰甘氨酸生成酶（FGE）的细胞系。示例性细胞系的产生和表征描述于与其同日提交的标题为“用于产生重组艾杜糖-2-硫酸酯酶的细胞（Cells for Producing Recombinant Iduronate-2-Sulfatase）”的美国临时申请中，其完整内容通过引用并入本文。简言之，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：5中显示的氨基酸序列的人甲酰甘氨酸生成酶（FGE）。

[0181] SEQ ID NO:2

[0182] ＞全长前体艾杜糖-2-硫酸酯酶

[0183] MPPPRTGRGLLWLGLVLSSVCVALGSETQANSTTDALNVLLIIVDDLRPSLGCYGDKLVRSPNIDQLASHSLLFQNAFAQQAVCAPSRVSFLTGRRPDTTRLYDFNSYWRVHAGNFSTIPQYFKENGYVTMSVGKVFHPGISSNHTDDSPYSWSFPPYHPSSEKYENTKTCRGPDGELHANLLCPVDVLDVPEGTLPDKQSTEQAIQLLEKMKTSASPFFLAVGYHKPHIPFRYPKEFQKLYPLENITLAPDPEVPDGLPPVAYNPWMDIRQREDVQALNISVPYGPIPVDFQRKIRQSYFASVSYLDTQVGRLLSALDDLQLANSTIIAFTSDHGWALGEHGEWAKYSNFDVATHVPLIFYVPGRTASLPFAGFKLFPYLDPFDSASQLMEPGRQSMDLVELVSLFPTLAGLAGLQVPPRCPVPSFHVELCREGKNLLKHFRFRDLEEDPYLPGNPRELIAYSQYPRPSDIPQWNSDKPSLKDIKIMGYSIRTIDYRYTVWVGFNPDEFLANFSDIHAGELYFVDSDPLQDHNMYNDSQGGDLFQLLMP

[0184] SEQ ID NO:5

[0185] 全长人FGE前体：

[0186] MAAPALGLVCGRCPELGLVLLLLLLSLLCGAAGSQEAGTGAGAGSLAGSCGCGTPQRPGAHGSSAAAHRYSREANAPGPVPGERQLAHSKMVPIPAGVFTMGTDDPQIKQDGEAPARRVTIDAFYMDAYEVSNTEFEKFVNSTGYLTEAEKFGDSFVFEGMLSEQVKTNIQQAVAAAPWWLPVKGANWRHPEGPDSTILHRPDHPVLHVSWNDAVAYCTWAGKRLPTEAEWEYSCRGGLHNRLFPWGNKLQPKGQHYANIWQGEFPVTNTGEDGFQGTAPVDAFPPNGYGLYNIVGNAWEWTSDWWTVHHSVEETLNPKGPPSGKDRVKKGGSYMCHRSYCYRYRCAARSQNTPDSSASNLGFRCAADRLPTMD

[0187] 在全长I2S酶合成后，25个氨基酸的信号肽被除去，并且可溶性成熟I2S酶从细胞分泌。

[0188] 将化学成分确定的培养基（无血清/无动物成分；AF）用于生物反应器工艺。

[0189] 减小个体收获材料的体积，并通过超滤/渗滤法交换缓冲液。将每个体收获物的材料（称为未纯化的批量(UPB)）在-50℃冷冻。下游纯化工艺始于未纯化批量的解冻和汇集，并且包括连续病毒灭活、阴离子交换(Capto Q)、混合模式（陶瓷羟基磷灰石）、阳离子交换(SP琼脂糖）和疏水性相互作用（苯基琼脂糖）色谱步骤，随后进行病毒过滤以及最终浓缩和渗滤步骤。具体地，该纯化过程使用Q、羟基磷灰石、SP和苯基色谱方式。去除常规用于I2S纯化工艺中的蛋白G色谱和尺寸排阻色谱。示例性步骤示于表3中。

[0190] 表3：纯化工艺的示例性步骤

[0191]

收获
UF/DF
冷冻贮存
解冻，汇集和深度过滤 ↓ 溶剂/去垢剂病毒灭活 ↓ 阴离子交换色谱 ↓ 混合模式色谱 ↓ ------------------- ↓ 稀释(1-1.2 倍) ↓ 阳离子交换色谱 ↓ 疏水相互作用色谱 ↓ ------------------- ↓ ------------------- ↓ 病毒去除过滤 ↓

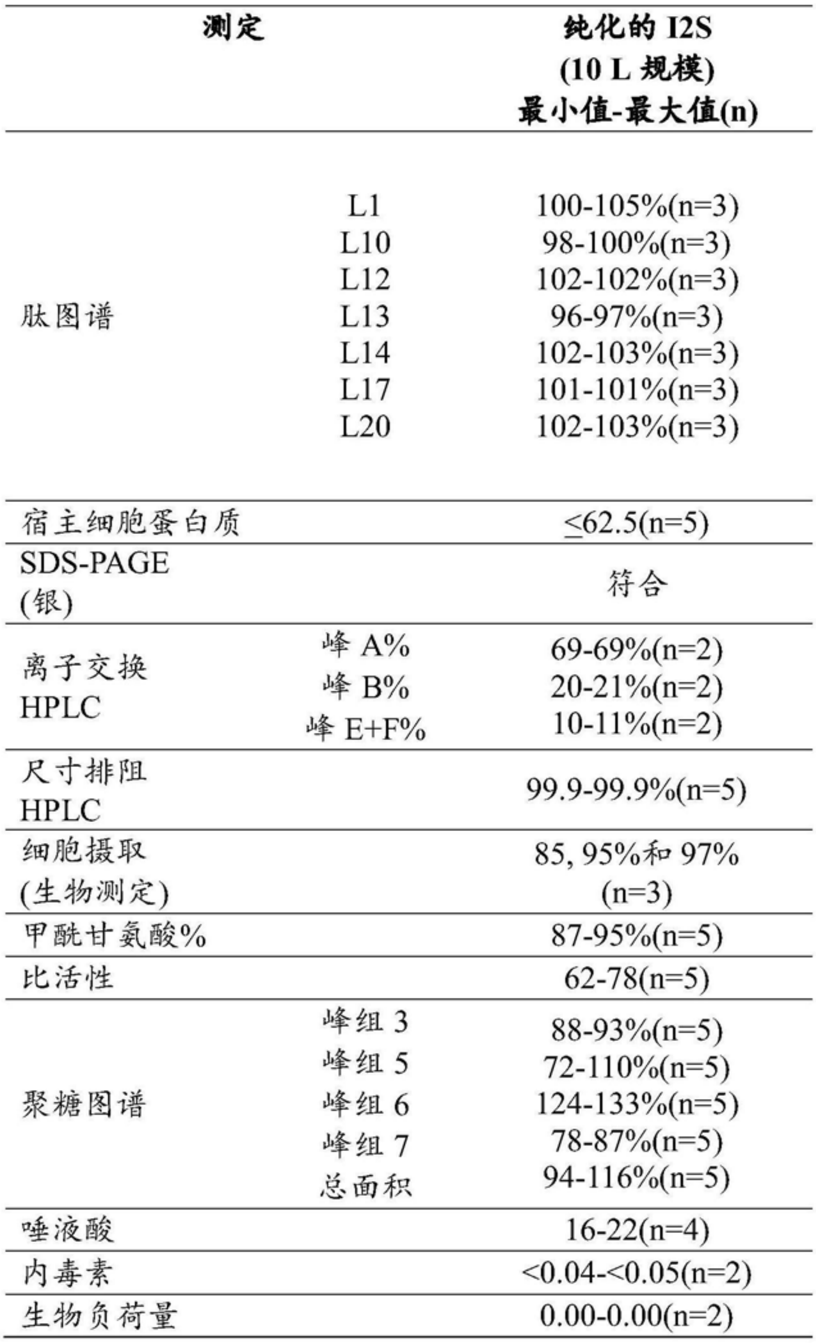
[0192]

UF/DF ↓ 原料药

[0193]　通过肽图谱、SDS-PAGE（银）、尺寸排阻 HPLC 评估纯化的 I2S 蛋白的纯度。使用标准方法测定酶特异性活性、甲酰甘氨酸含量、唾液酸含量、聚糖图谱、电荷特征谱。示例性结果示于表 4 中。

[0194]　表 4：纯化的重组 I2S 蛋白的分析

[0195]



测定		纯化的 I2S (10 L 规模) 最小值-最大值(n)
肽图谱	L1	100-105%(n=3)
	L10	98-100%(n=3)
	L12	102-102%(n=3)
	L13	96-97%(n=3)
	L14	102-103%(n=3)
	L17	101-101%(n=3)
	L20	102-103%(n=3)
宿主细胞蛋白质		≤62.5(n=5)
SDS-PAGE (银)		符合
离子交换 HPLC	峰 A%	69-69%(n=2)
	峰 B%	20-21%(n=2)
	峰 E+F%	10-11%(n=2)
尺寸排阻 HPLC		99.9-99.9%(n=5)
细胞摄取 (生物测定)		85, 95%和 97% (n=3)
甲酰甘氨酸%		87-95%(n=5)
比活性		62-78(n=5)
聚糖图谱	峰组 3	88-93%(n=5)
	峰组 5	72-110%(n=5)
	峰组 6	124-133%(n=5)
	峰组 7	78-87%(n=5)
	总面积	94-116%(n=5)
唾液酸		16-22(n=4)
内毒素		<0.04-<0.05(n=2)
生物负荷量		0.00-0.00(n=2)

[0196] 示例性肽图谱相较于商购可得I2S参考示于图2中。示例性SDS-PAGE（银）分析结果示于图3中。通常情况下，通过使用本文中描述的工艺，原料药的HCP浓度(DS)<100ppm，满足在许多市场（包括美国）中所需的<100ppm的规范。DS的SEC ≥ 99.5%，也满足许多市场的当前>99.3%的营销规范要求。示例性电荷特征谱示于图4中。示例性聚糖图谱示于图5中。具体地，纯化的I2S的聚糖图谱包括七个的峰组，根据递增量的来源于唾液酸和甘露糖-6-磷酸残基的负电荷洗脱，按照洗脱的顺序代表中性、单、二唾液酸化、单磷酸化、三唾液酸化和混合（单唾液酸化和封端M6P）、四唾液酸化和混合（二唾液酸化和封端M6P）以及二磷酸化聚糖。

[0197] 综上所述，本实施例证明，简化的四柱纯化工艺可用于成功地纯化在无动物成分培养基中大规模产生的重组I2S。

[0198] 实施例2：重组I2S AF的收获和病毒灭活稳定性研究

[0199] 本研究的目的是评价温度保持时间和冷冻-解冻循环对重组I2S澄清收获物的稳定性的影响。

[0200] 将澄清的收获样品在环境温度和2-8℃贮存达7天，并将灭活病毒的UPB样品在环境温度下保持达24小时。将澄清收获物的冻融样品在-20℃、-50℃、-80℃下冷冻，并经历冷冻-解冻达3个循环。使用Western印迹、SEC HPLC和活性测定精确计量稳定性。

[0201] 使用具有离心保留装置的B.Braun 20L生物反应器和期望的泌水率，通过CCPD从2D细胞系产生I2S-AF收获材料。对于温度保持研究，将每一个澄清的收获物在环境温度和2-8℃下贮存，并在选择的保持时间上取样。采样量和保持时间列表5中。将冷冻-解冻样品于-20℃、-50℃和-80℃下贮存，并使用25℃水浴解冻。

[0202] 表5. 澄清的收获物保持点稳定性

[0203]

[0204]

	样品	保持温度	保持时间(天)
澄清的收获物12	15 x 0.5 mL	2-8℃	T=0、24小时、76小时、120小时、168小时
	15 x 0.5 mL	环境温度	T=0、24小时、76小时、120小时、168小时
	9 x 0.5 mL	-20℃, -50℃和-80℃	冷冻/解冻1、2和3
澄清的收获物18	15 x 0.5 mL	2-8℃	T=0、24小时、76小时、120小时、168小时
	15 x 0.5 mL	环境温度	T=0、24小时、76小时、120小时、168小时
	9 x 0.5 mL	-20℃, -50℃和-80℃	冷冻/解冻1、2和3

[0205] 病毒灭活步骤在上样第一柱之前的未纯化的批量步骤中发生。通过澄清的收获物的浓缩和缓冲交液换产生UPB。使用Pall 1sq.ft.Centramate系统进行UF/DF，并且将缓冲液交换成10mM MES，155mM NaCl，pH＝6.5。病毒灭活步骤添加1％ Tween 80和0.3％ TnBP，对于每一个时间点使用Durapore注射器过滤器进行过滤。在表6中所列的每一个时间点取样，将其在-80℃下冷冻。通过Western印迹和活性(4-MU测定)测试来自澄清的收获保持点和冷冻-解冻研究的样品。通过SEC HPLC测试来自病毒灭活的UPB样品的纯度。来自表5中的收获物12和18的保持点活性结果显示，对于两种收获物，在环境温度和2-8℃下在达7天的贮存中没有显著的变化。对于在-20℃、-50℃和-80℃贮存的，经历达3个冷冻-解冻循环的收获物12活性没有显著变化。

[0206] 表6. 未纯化的批量的病毒灭活

[0207]

	样品	保持温度	保持时间（天）
病毒灭活	9x 0.5mL	环境温度,控制	T ＝ 0,6 小时,24 小时
	9x 0.5mL	环境温度,病毒灭活	T ＝ 0,6 小时,24 小时

[0208] 关于病毒灭活 UPB 步骤的稳定性的活性和 SEC-HPLC 描述于图 6 和 7 中。这显示，基于长达 24 小时的活性和纯度,病毒灭活的稳定性没有问题。

[0209] 总之,基于本文中描述的稳定性分析,可在 2-8℃下贮存澄清的收获物（例如，长达 7 天）而无收获物质量的显著变化。澄清的收获物可经历多个冷冻 - 解冻循环并于 -20℃、-50℃、-80℃的温度下贮存,而无稳定性的显著变化。基于 SEC HPLC 纯度结果，在 UPB 步骤中进行的病毒灭活可在环境温度下进行（例如,长达 24 小时）而无活性和纯度的变化。

[0210] 实施例 3 :无动物成分 IL CD 培养基确认运行的纯化和分析

[0211] 本研究的目的是从 I2S-AF 的汇集收获物进行纯化和表征原料药,所述 I2S-AF 是使用化学成分确定的培养基在无动物成分灌注中产生的。

[0212] 本研究评估 I2S-AF 纯化工艺性能和从化学成分确定的培养基生物反应器产生的原料药 (DS)。

[0213] 细胞培养

[0214] I2S-AF 材料从如实施例 1 中描述的表达 I2S 和甲酰甘氨酸生成酶 (FGE)) 的细胞系 2D 产生。使用化学成分明确的无血清培养基,在 1L Das Gip 旋转过滤器生物反应器中于 CCPD 中产生材料。将来自每一个澄清的收获物 (HI-21) 的单个袋子在 -20℃下冷冻,在 2-8℃解冻过夜。将等体积的每一个澄清的收获物汇集以代表整个收获汇集物,随后进行 0.2μm 过滤,并用 30kD 的 Pall Omega Centramate 盒（具有 1ft^{2}的总膜面积）进行浓缩。将未纯化批量 (UPB) 进行 0.2um 过滤,并在使用前进行冷冻。

[0215] 纯化

[0216] 示例性柱规格和上样描述于表 7 中。通过滴定以 3g/L 的靶上样至 Q 琼脂糖。将先前的柱洗脱液 100％地上样至随后的柱,并且无材料被去除。

[0217] 表 7. 柱和上样规范

[0218]

柱	柱尺寸(cm x cm)	柱体积(mL)	柱载荷(g/L 树脂，按 I2S 计)	柱载荷 (mg)
Q 琼脂糖	2.6 x 25	133	3	399
HA II 型, 80 μm	1.6 x 30	60	5.5	330
苯基琼脂糖	1.6 x 23	46	5.6	258

[0219] 利用来自汇集收获物 1 至 21（来自生物反应器）的 UPB 进行一个纯化运行。将 UPB 在 2-8℃解冻过夜,并从每一个收获物等体积汇集 UPB。

[0220] 单个柱工艺步骤和缓冲制剂可见于表 8-11 中。使用 0.2um 瓶过滤器系统过滤汇集的 UPB,使用 1M 醋酸钠将其调整至 pH6.5,并在上样至 Q 琼脂糖 FF 柱之前,用 5M 氯化钠

将电导率调整至16mS/cm。在上样至HA柱之前，使用0.25M NaPO$_4$(pH 5.5)将Q琼脂糖洗脱液调整至0.001M NaPO$_4$，并用0.22um PES瓶顶过滤器进行过滤。用5M NaCl将HA洗脱液的电导率调整至1.55M NaCl，并且用1M醋酸钠将pH调整至pH 5.5。调整时间为约1小时。在上样至苯基琼脂糖柱上之前，使用0.22um的PES瓶顶过滤器过滤经调整的汇集液。将苯基洗脱液浓缩4倍，和渗滤6倍至0.02M NaPO$_4$，0.137M NaCl，pH 6.0中。将渗滤产物调整至2.0g/L，并用0.2％聚山梨酯20配制，以产生模拟原料药。产生DS的H1-20的模拟汇集物以用于额外表征。

[0221]　表8.Q琼脂糖FF色谱的示例性工艺细节

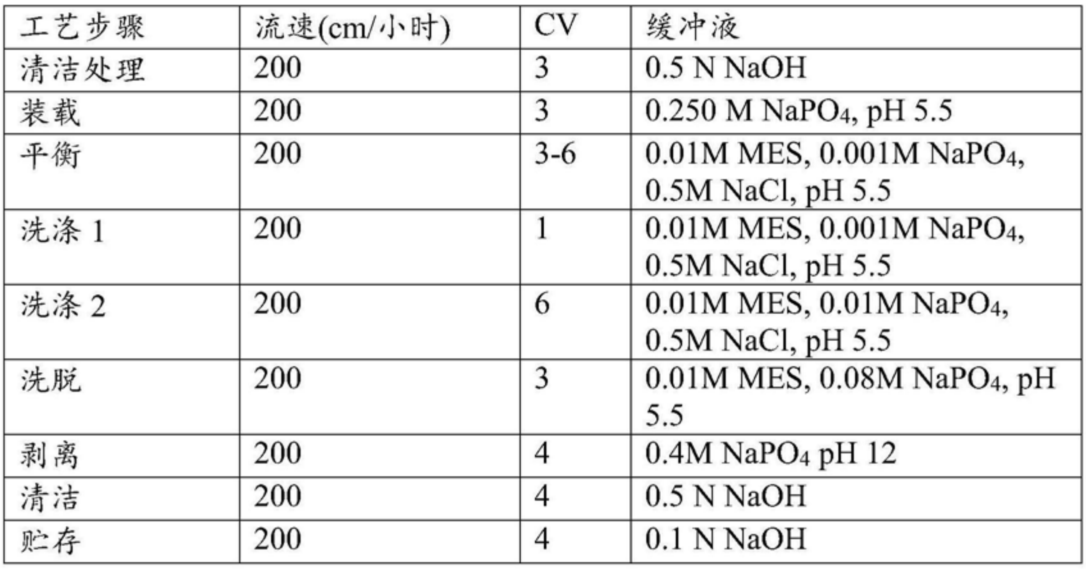
[0222]

工艺步骤	流速（cm/小时）	CV	缓冲液
清洁处理	150	3	0.5N NaOH
平衡	150	4	0.01M MES，0.155M NaCl，ph 6.5
洗涤1	150	2	0.01M MES，0.155M NaCl，ph 6.5
洗涤2	150	3	0.01M MES，0.155M NaCl，ph 5.5
洗脱	150	3	0.01M MES，0.50M NaCl，ph 5.5
清洁/剥离	150	4	1.0M NaOH，2M NaCl
贮存	150	4	0.0N NaOH

[0223]

[0224]　表9.HA色谱的示例性工艺细节

[0225]



工艺步骤	流速(cm/小时)	CV	缓冲液
清洁处理	200	3	0.5 N NaOH
装载	200	3	0.250 M NaPO$_4$, pH 5.5
平衡	200	3-6	0.01M MES, 0.001M NaPO$_4$, 0.5M NaCl, pH 5.5
洗涤1	200	1	0.01M MES, 0.001M NaPO$_4$, 0.5M NaCl, pH 5.5
洗涤2	200	6	0.01M MES, 0.01M NaPO$_4$, 0.5M NaCl, pH 5.5
洗脱	200	3	0.01M MES, 0.08M NaPO$_4$, pH 5.5
剥离	200	4	0.4M NaPO$_4$ pH 12
清洁	200	4	0.5 N NaOH
贮存	200	4	0.1 N NaOH

[0226]　表10.苯基琼脂糖色谱的示例性工艺细节

[0227]

工艺步骤	流速（cm/ 小时）	CV	缓冲液
清洁处理	150	3	0.5N NaOH
平衡	150	4-6	0.02M MES，1.5M NaCl，pH 5.5
洗涤	150	2	0.02M MES，1.5M NaCl，pH 5.5
洗脱	150	3	0.02M MES，0.2M NaCl，pH 5.5
水洗涤	150	3	RO/ 去离子水
乙醇洗涤	150	3	20％乙醇
清洁	150	3	0.5N NaOH
贮存	150	3	0.01N NaOH

[0228] 表 11. 苯基洗脱汇集物的示例性渗滤

[0229]

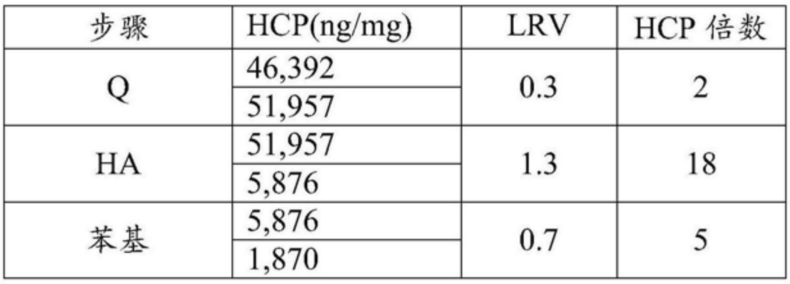
过滤装置	Centricon Plus 70
渗滤缓冲液	0.02M $NaPO_4$，0.137M NaCl，pH 6.0
渗滤体积	6X-8X

[0230] 通过 ELISA 测定的 HCP 的过程中纯度

[0231] 表 12 描述每一个步骤的过程中 HOP 去除。对于 HA 步骤中的大部分去除，过程中 HCP 结果很高。

[0232] 表 12. 过程中 HCP 去除

[0233]



步骤	HCP(ng/mg)	LRV	HCP 倍数
Q	46,392	0.3	2
	51,957		
HA	51,957	1.3	18
	5,876		
苯基	5,876	0.7	5
	1,870		

[0234] 原料药表征

[0235] 示例性原料药大量释放的结果列于表 13 中。如可看到的，原料药在纯材料中具有高比活性和％ FG。示例性原料药属性表征示于表 13 中。在终 UF/DF 步骤中 HCP 从 1,870ng/mg 减少至 372ng/mg。

[0236] 表 13. 示例性原料药的大量释放

[0237]

DS 大量释放	1L CD 培养基 (I2S-AF)
%FG	94%
聚糖图谱	
组 3	99%
组 5	89%
组 6	104%
组 7(2-M6P)	95%
总面积	107%
唾液酸	17
内化	83%
SEC-HPLC	99.9%
比活性(U/mg)	82
IEX HPLC	
A(%)	64%
B(%)	23%
A+B	87%
E+F	0%
宿主细胞蛋白质	372
细胞摄取	98

[0238] 实施例 4. 纯化的重组 I2S 酶的生理化学和生物学表征

[0239] 本实施例的目的是进行使用上述方法纯化的重组 I2S 蛋白的详细表征。

[0240] SDS-PAGE

[0241] 关于实验，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。收集样品，并使用上述方法进行纯化。通过 SDS-PAGE 分析纯化的 I2S 酶，并用银染进行处理以使其可见。示例性结果示于图 8 中。如可从图 8 中看到的，使用本文中描述的方法的纯化的重组 I2S 蛋白显示可与使用标准方法纯化的 I2S 参考样品可比较的带型模式。

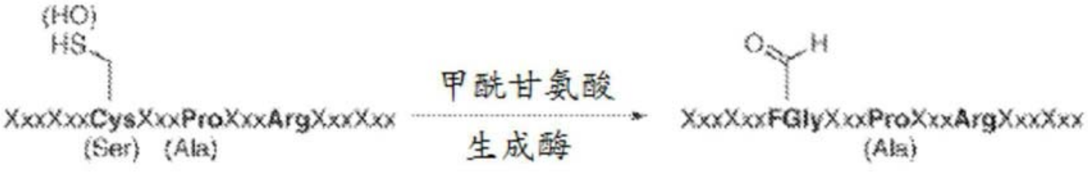
[0242] 肽图谱

[0243] 使用如上所述方法纯化由 I2S-AF 2D 细胞系产生的重组 I2S 蛋白。使纯化的重组 I2S 和参考人 I2S 的样品各自经历蛋白水解消化，并通过 HPLC 分析进行检查。相较于参考 I2S 的肽图谱的示例性肽图谱示于图 9 中。

[0244] 甲酰甘氨酸转化百分比

[0245] 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：

[0246]



[0247] 因此，甲酰甘氨酸转化的百分比 (% FG) 可以使用下列公式计算：

[0248]

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

[0249] 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。

[0250] 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）将纯化的重组 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。示例性结果示于表 14 中。

[0251] 聚糖图谱－甘露糖 -6 磷酸和唾液酸含量

[0252] 测定纯化的重组 I2S 蛋白的聚糖和唾液酸的组成。使用阴离子交换色谱法产生聚糖图谱来进行聚糖组合物的定量。如下所述，在本文中描述的条件下纯化的重组 I2S 的聚糖图谱由七个峰组组成，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- 磷酸糖产生）洗脱。简言之，使用如下方式处理来自无血清细胞培养物（I2S-AF2D 无血清和 I2S-AF 4D 无血清）的纯化的重组 I2S 和参考重组 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 和参考 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 10 中显示的，由无血清培养基纯化的 I2S 的聚糖图谱显示构成中性、单、二唾液酸化、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）。示例性聚糖图谱示于图 10 中。

[0253] 根据唾液酸标准的线性回归分析计算每一个重组 I2S 样品中的平均唾液酸含量（每摩尔蛋白质的唾液酸摩尔数）。使用 PeakNet 6 软件使每一个色谱图运行可视化。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：

[0254]

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

[0255] 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）。

[0256] 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：

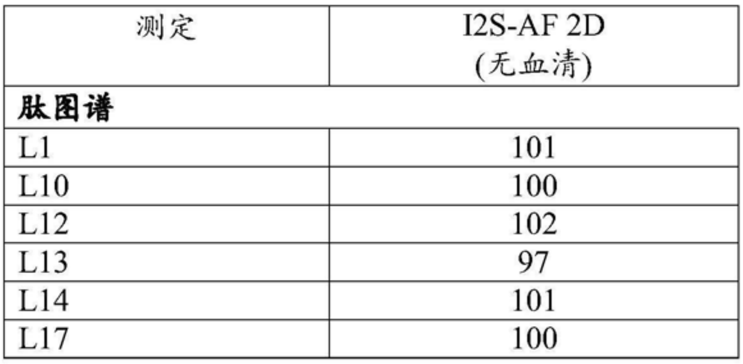
[0257]

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

[0258]　表示由 I2S-AF 2D 或 4D 细胞系纯化的重组 I2S 上的唾液酸含量的示例性数据示于表 14 中。

[0259]　表 14：从无血清细胞培养基物纯化的 I2S 的示例性特征

[0260]



测定	I2S-AF 2D (无血清)
肽图谱	
L1	101
L10	100
L12	102
L13	97
L14	101
L17	100

[0261]

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
峰 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(n=13)
聚糖图谱	
单唾液酸化	105
二唾液酸化	93
单磷酸化	139
三唾液酸化	89
四唾液酸化	125
二磷酸化	95
唾液酸(mol/mol)	20

[0262]　比活性

[0263]　使用体外硫酸酯释放测定或 4-MUF 测定使用本文所述的方法纯化的重组 I2S 酶的比活性。

[0264]　体外硫酸酯释放测定

[0265]　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 (pH6) 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 30μL 反应体积中以 0.3μg I2S/100μg 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 14 中。

[0266] 4-MUF 测定

[0267] 还可使用基于荧光的 4-MUF 测定来分析纯化的重组 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：

[0268]

$$\%CV=\frac{\text{原始荧光值的标准差}(N=3)}{\text{平均荧光值}}X100\%$$

[0269] 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：

[0270]

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

[0271] CFU ＝负修正平均荧光

[0272] DF - 稀释因子

[0273] 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。

[0274] 电荷特征谱

[0275] 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-HCl，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。显示针对从2D和4D细胞系纯化的重组I2S观察到的电荷特征谱的示例性结果示于图11中。

[0001]

序列表

<110> 夏尔人类遗传性治疗公司

<120> 艾杜糖-2-硫酸酯酶的纯化

<130> 2006685-0342

<150> 61/666,733
<151> 2012-06-29

<160> 5

<170> PatentIn 3.5版

<210> 1
<211> 525
<212> PRT
<213> 智人

<400> 1

```
Ser Glu Thr Gln Ala Asn Ser Thr Thr Asp Ala Leu Asn Val Leu Leu
1               5                   10                  15

Ile Ile Val Asp Asp Leu Arg Pro Ser Leu Gly Cys Tyr Gly Asp Lys
            20                  25                  30

Leu Val Arg Ser Pro Asn Ile Asp Gln Leu Ala Ser His Ser Leu Leu
        35                  40                  45

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
    130                 135                 140
```

[0002]

```
Thr Cys Arg Gly Pro Asp Gly Glu Leu His Ala Asn Leu Leu Cys Pro
145                 150                 155                 160

Val Asp Val Leu Asp Val Pro Glu Gly Thr Leu Pro Asp Lys Gln Ser
                165                 170                 175

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
    210                 215                 220

Ala Pro Asp Pro Glu Val Pro Asp Gly Leu Pro Pro Val Ala Tyr Asn
225                 230                 235                 240

Pro Trp Met Asp Ile Arg Gln Arg Glu Asp Val Gln Ala Leu Asn Ile
                245                 250                 255

Ser Val Pro Tyr Gly Pro Ile Pro Val Asp Phe Gln Arg Lys Ile Arg
            260                 265                 270

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
                325                 330                 335

Tyr Val Pro Gly Arg Thr Ala Ser Leu Pro Glu Ala Gly Glu Lys Leu
            340                 345                 350

Phe Pro Tyr Leu Asp Pro Phe Asp Ser Ala Ser Gln Leu Met Glu Pro
        355                 360                 365
```

[0003]

Gly Arg Gln Ser Met Asp Leu Val Glu Leu Val Ser Leu Phe Pro Thr
370 375 380

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
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<213> 智人

[0008]

```
<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

Leu Glu Lys Met Lys Thr Ser Ala Ser Pro Phe Phe Leu Ala Val Gly
```

[0009]

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> 374
<212> PRT
<213> 智人

<400> 5

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

[0010]

```
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

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
```

[0011]

```
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

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
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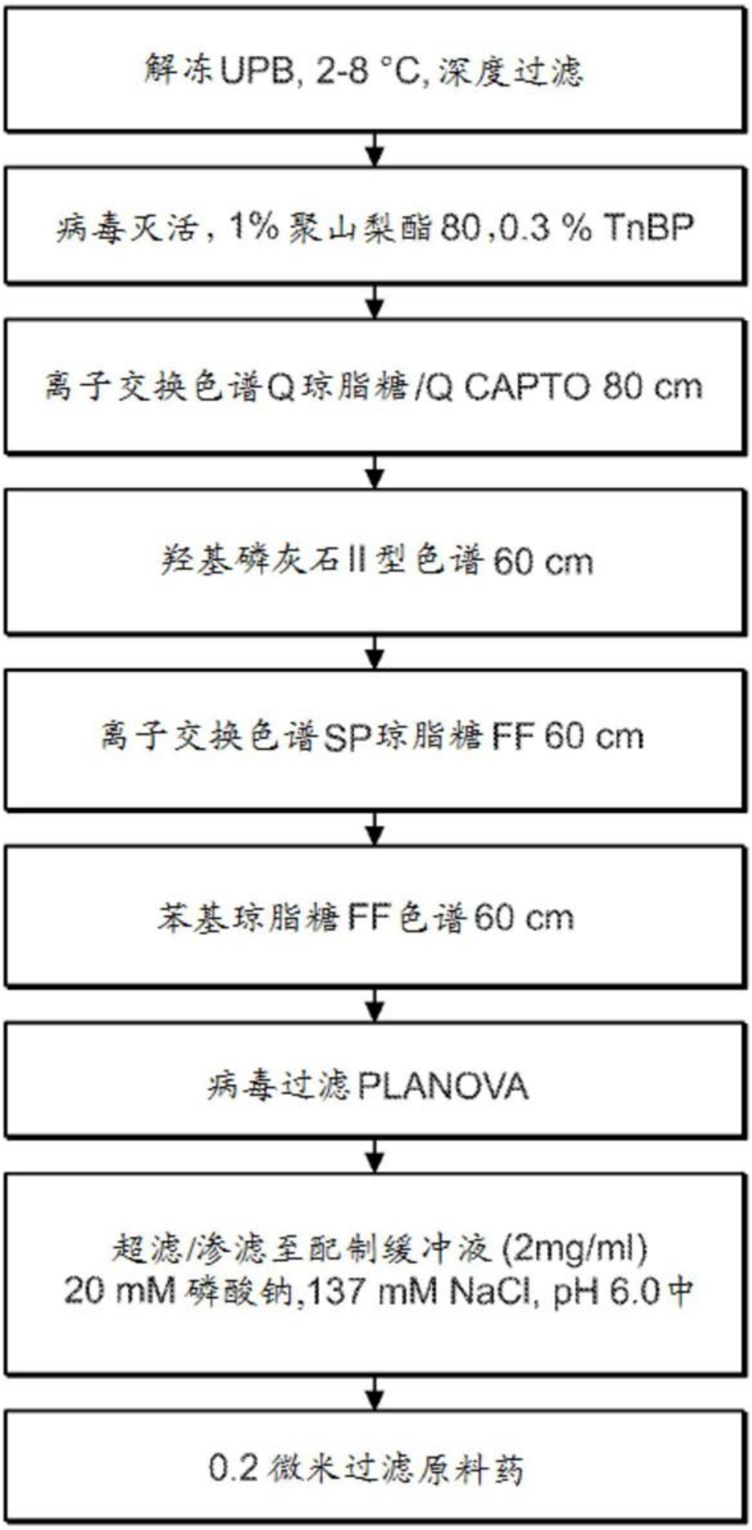

解冻UPB, 2-8 °C, 深度过滤
病毒灭活，1% 聚山梨酯 80, 0.3 % TnBP
离子交换色谱Q琼脂糖/Q CAPTO 80 cm
羟基磷灰石II型色谱 60 cm
离子交换色谱SP琼脂糖 FF 60 cm
苯基琼脂糖 FF 色谱 60 cm
病毒过滤 PLANOVA
超滤/渗滤至配制缓冲液 (2mg/ml)
20 mM 磷酸钠, 137 mM NaCl, pH 6.0中
0.2 微米过滤原料药

图 1

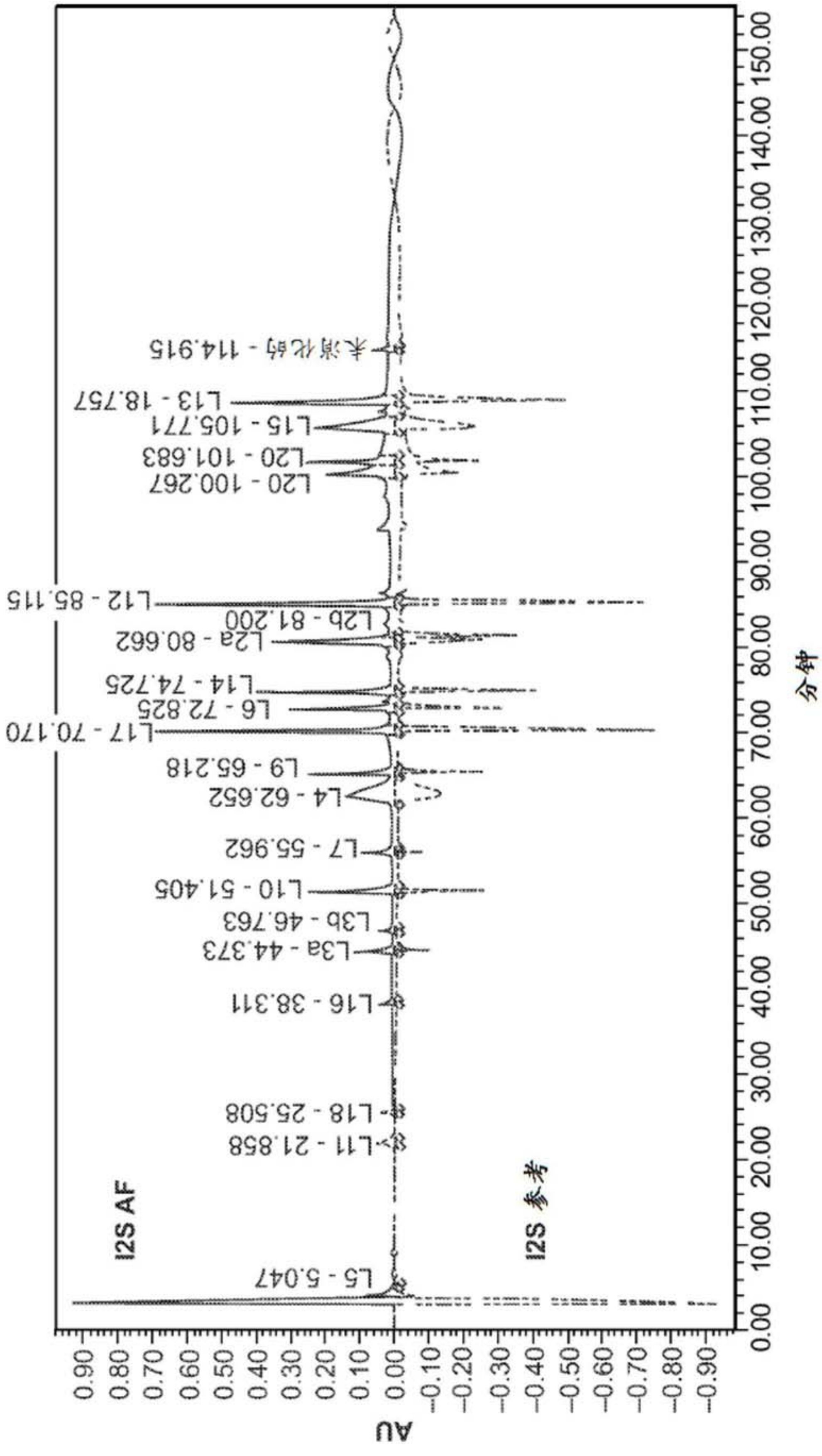
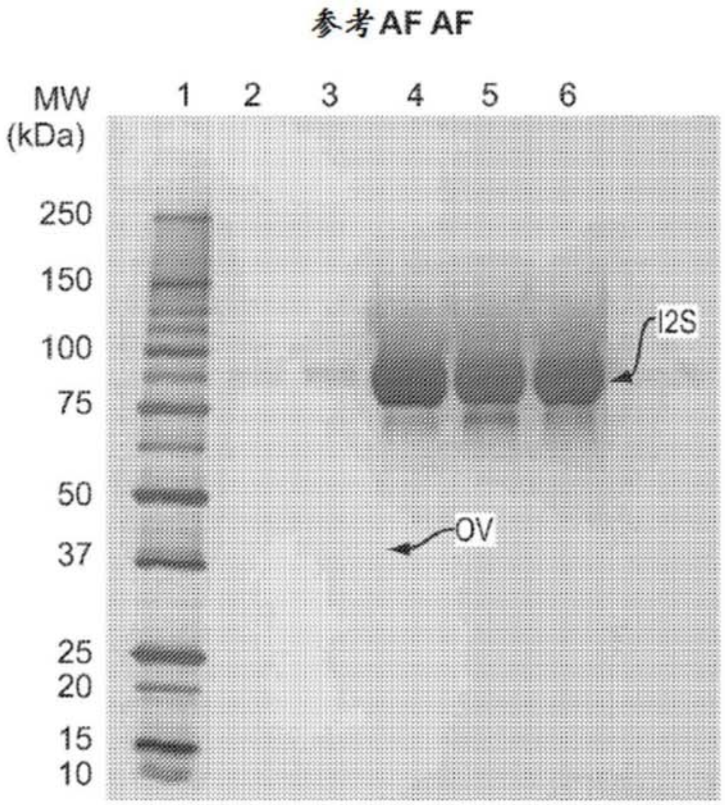

I2S AF
I2S 参考
L5 - 5.047
L11 - 21.858
L18 - 25.508
L16 - 38.311
L3a - 44.373
L3b - 46.763
L10 - 51.405
L7 - 55.962
L4 - 62.652
L9 - 65.218
L17 - 70.170
L6 - 72.825
L14 - 74.725
L2a - 80.662
L2b - 81.200
L12 - 85.115
L20 - 100.267
L20 - 101.683
L15 - 105.771
L13 - 18.757
未消化的 - 114.915
AU
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
-0.20
-0.30
-0.40
-0.50
-0.60
-0.70
-0.80
-0.90
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
150.00
分钟

图 2



泳道	上样样品	PAD ID	上样体积 μl	总的μg
1	精度加蛋白质标准 (未染色的)	▨	3 μl	▨
2	测定对照 #1	▨	20	各自8 μg, OV末端参考
3	测定对照 #2	▨	20	各自18 μg, OV末端参考
4	参考标准	▨	20	8
5	RDSF 11-101 模拟物DS	11AD0545-1	20	8
6	RDSF 11-105 模拟物DS	11AD0545-2	20	8

图 3

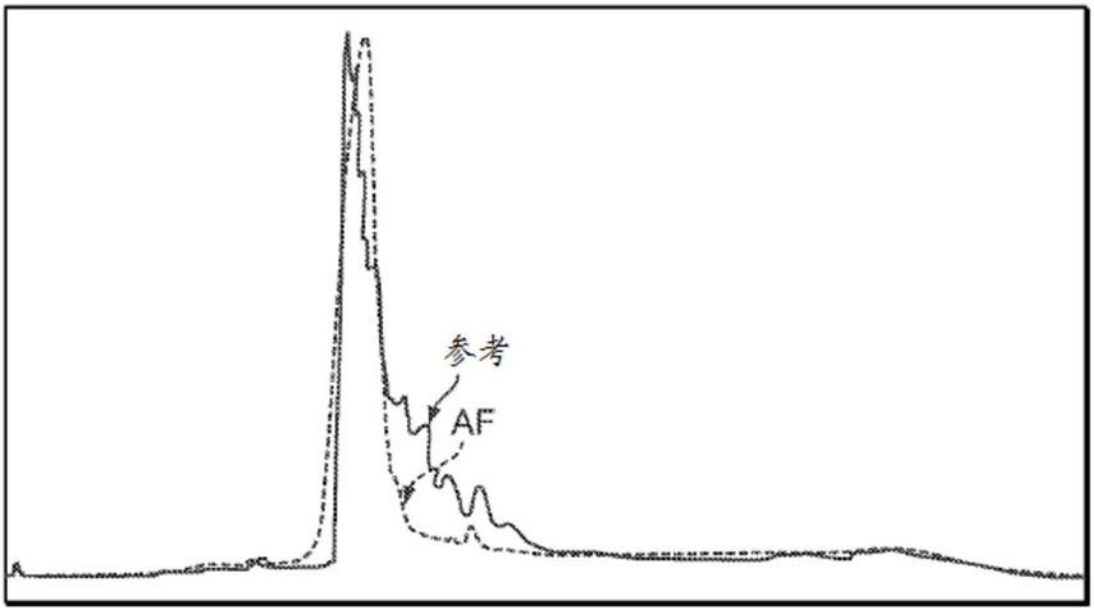


图 4

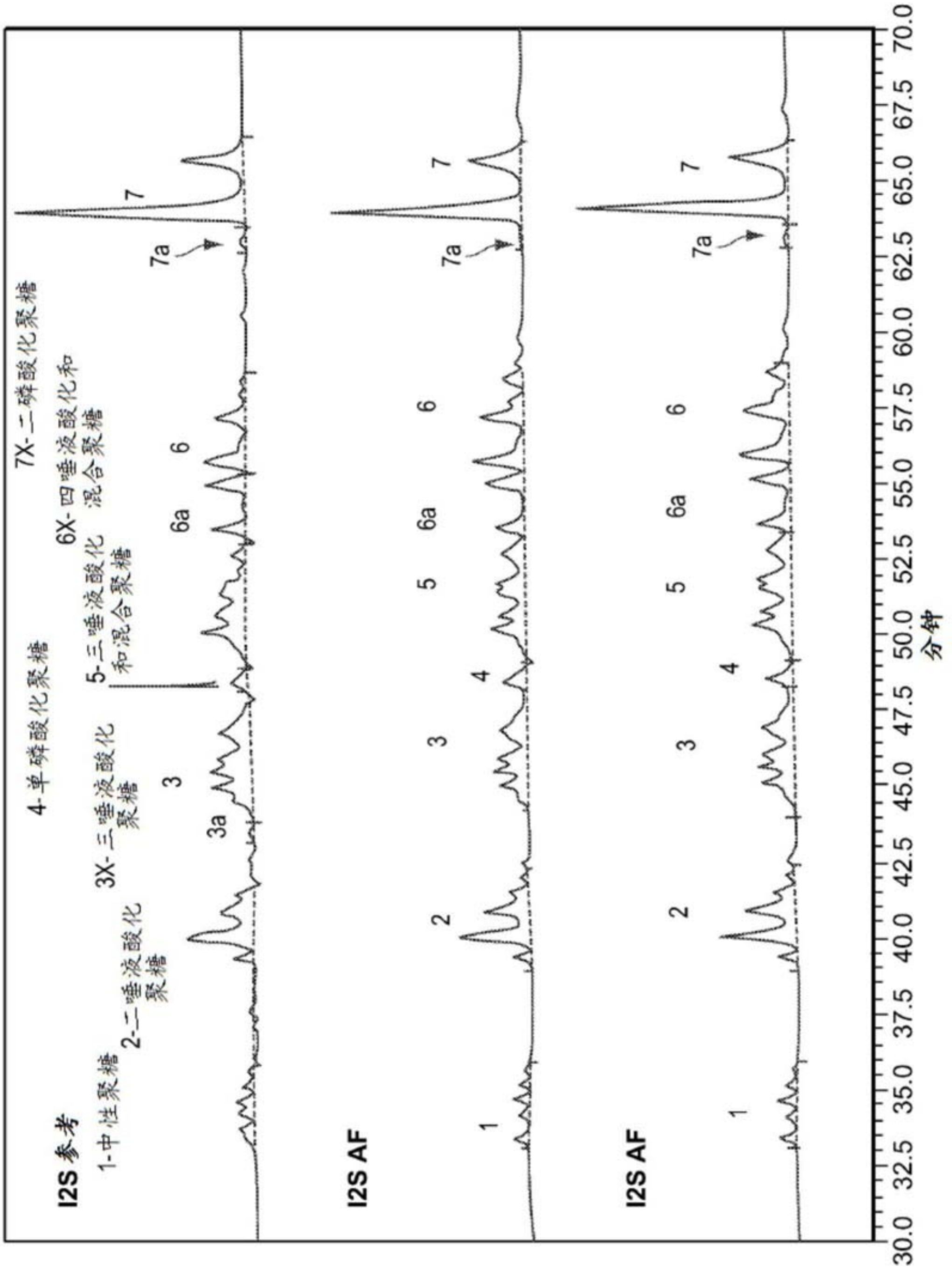

I2S 参考
1-中性聚糖
2-二唾液酸化聚糖
3X-三唾液酸化聚糖
4-单磷酸化聚糖
5-三唾液酸化和混合聚糖
6X-四唾液酸化和混合聚糖
7X-二磷酸化聚糖
1
2
3a
3
6a
6
7a
7
I2S AF
1
2
3
4
5
6a
6
7a
7
I2S AF
1
2
3
4
5
6a
6
7a
7
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
70.0
分钟

图 5

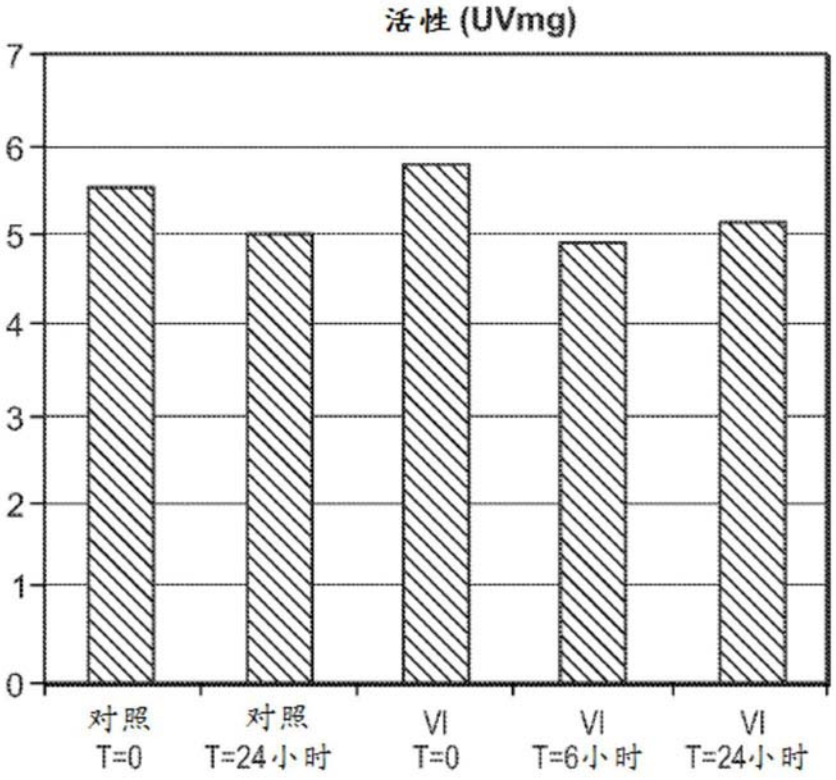

活性 (UVmg)
7
6
5
4
3
2
1
0
对照 T=0
对照 T=24小时
VI T=0
VI T=6小时
VI T=24小时

图 6

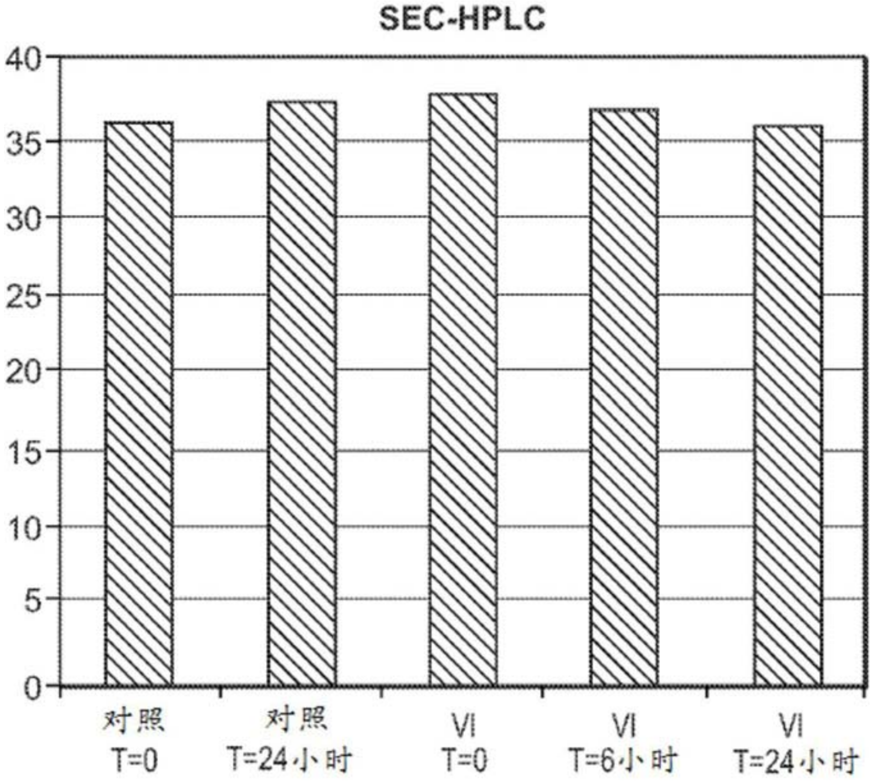
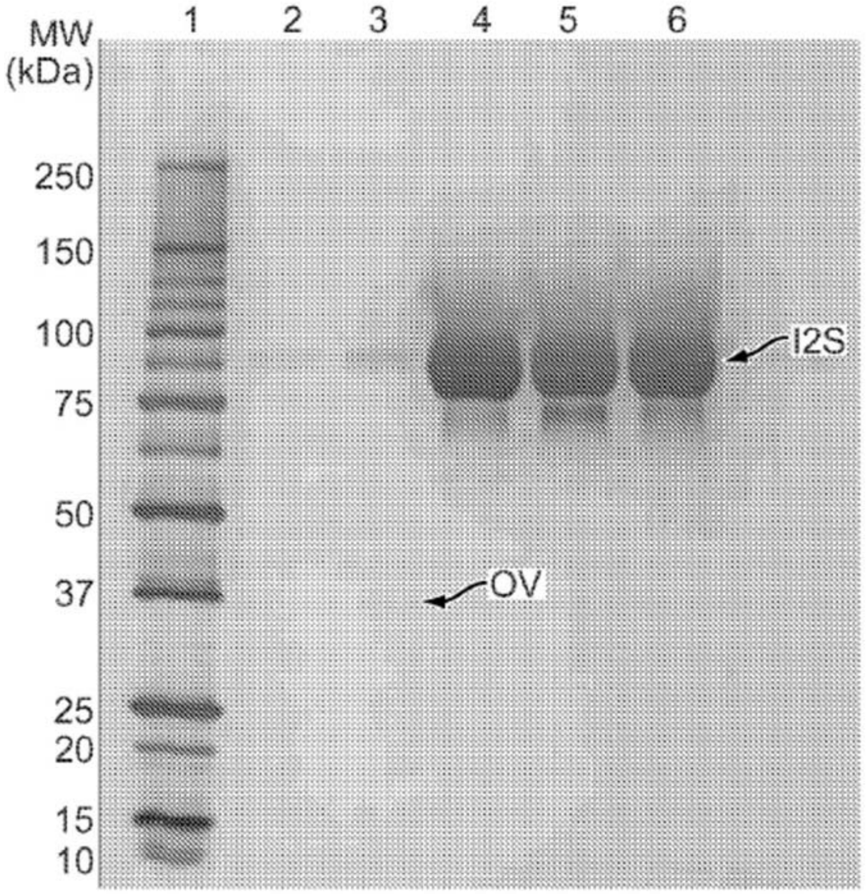

SEC-HPLC
40
35
30
25
20
15
10
5
0
对照 T=0
对照 T=24小时
VI T=0
VI T=6小时
VI T=24小时

图 7



泳道	上样样品	上样体积	总的µg
1	蛋白质标准	3 µl	
2	测定对照 #18	20 µl	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 8

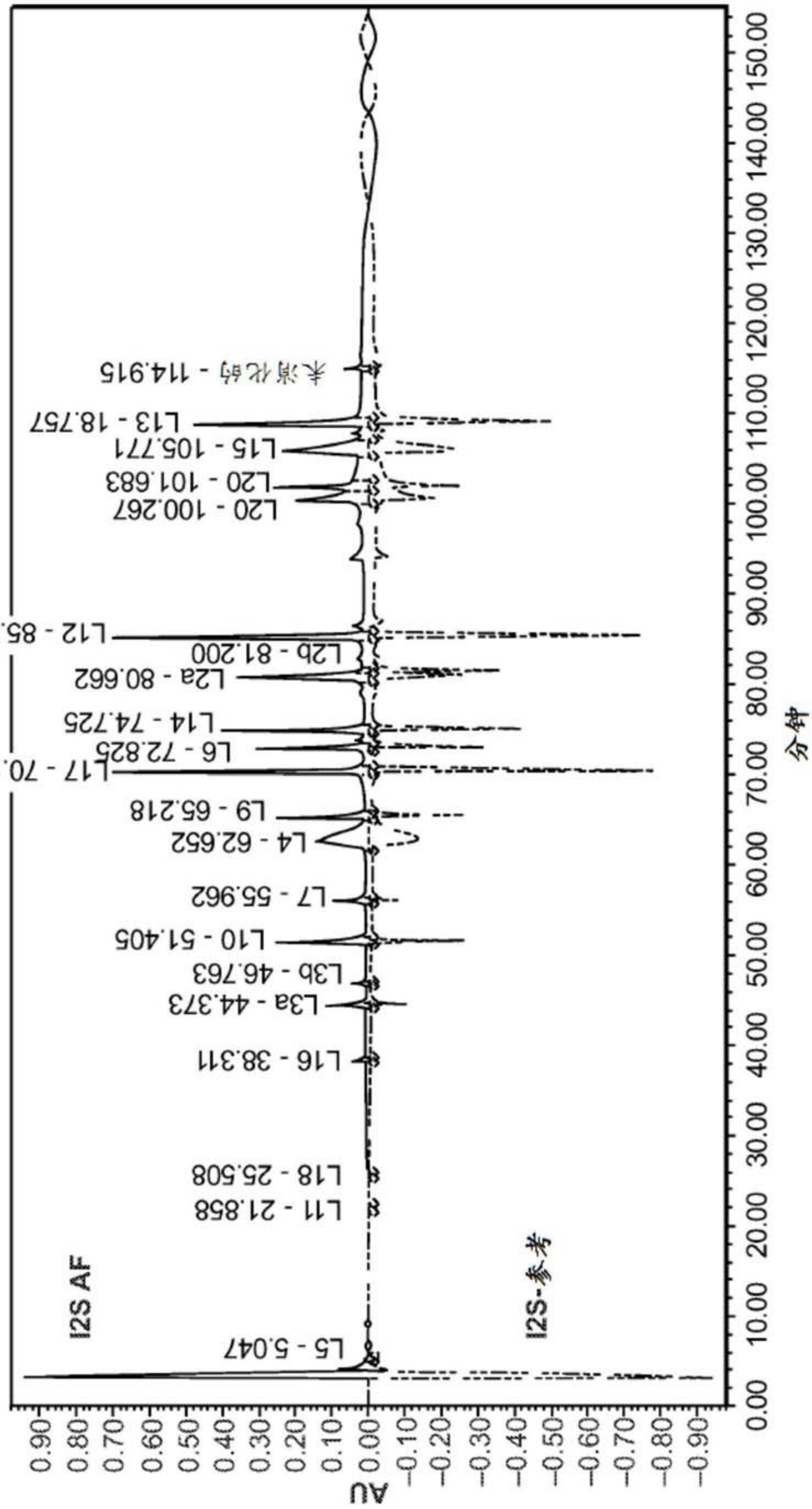

I2S AF
I2S-参考
L5 - 5.047
L11 - 21.858
L18 - 25.508
L16 - 38.311
L3a - 44.373
L3b - 46.763
L10 - 51.405
L7 - 55.962
L4 - 62.652
L9 - 65.218
L17 - 70.170
L6 - 72.825
L14 - 74.725
L2a - 80.662
L2b - 81.200
L12 - 85.115
L20 - 100.267
L20 - 101.683
L15 - 105.771
L13 - 18.757
未消化的 - 114.915
AU
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
−0.10
−0.20
−0.30
−0.40
−0.50
−0.60
−0.70
−0.80
−0.90
分钟
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
150.00

图 9

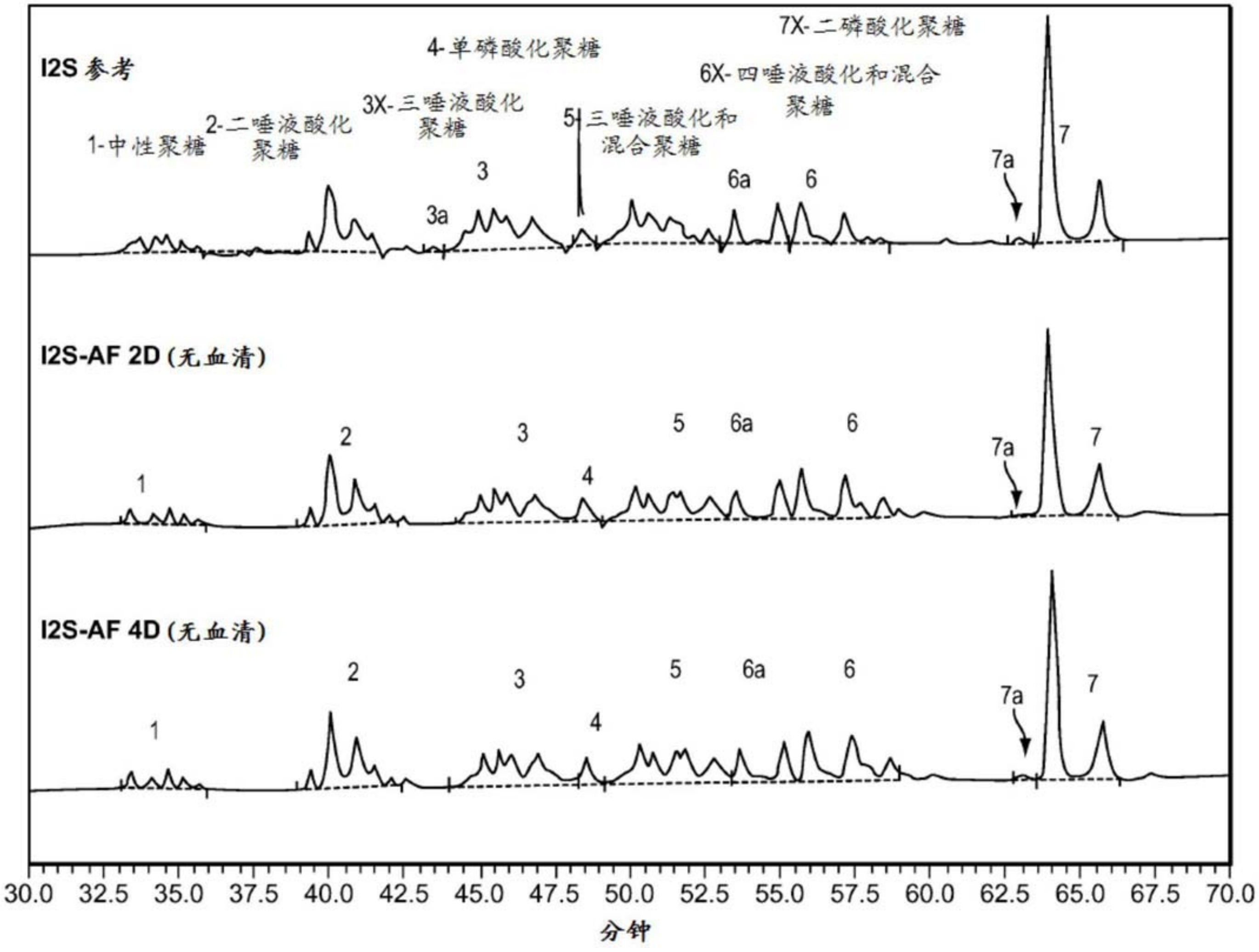

I2S 参考
1-中性聚糖
2-二唾液酸化聚糖
3X-三唾液酸化聚糖
4-单磷酸化聚糖
5-三唾液酸化和混合聚糖
6X-四唾液酸化和混合聚糖
7X-二磷酸化聚糖
3a
3
5
6a
6
7a
7
I2S-AF 2D (无血清)
1
2
3
4
5
6a
6
7a
7
I2S-AF 4D (无血清)
1
2
3
4
5
6a
6
7a
7
30.0
32.5
35.0
37.5
40.0
42.5
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
70.0
分钟

图 10

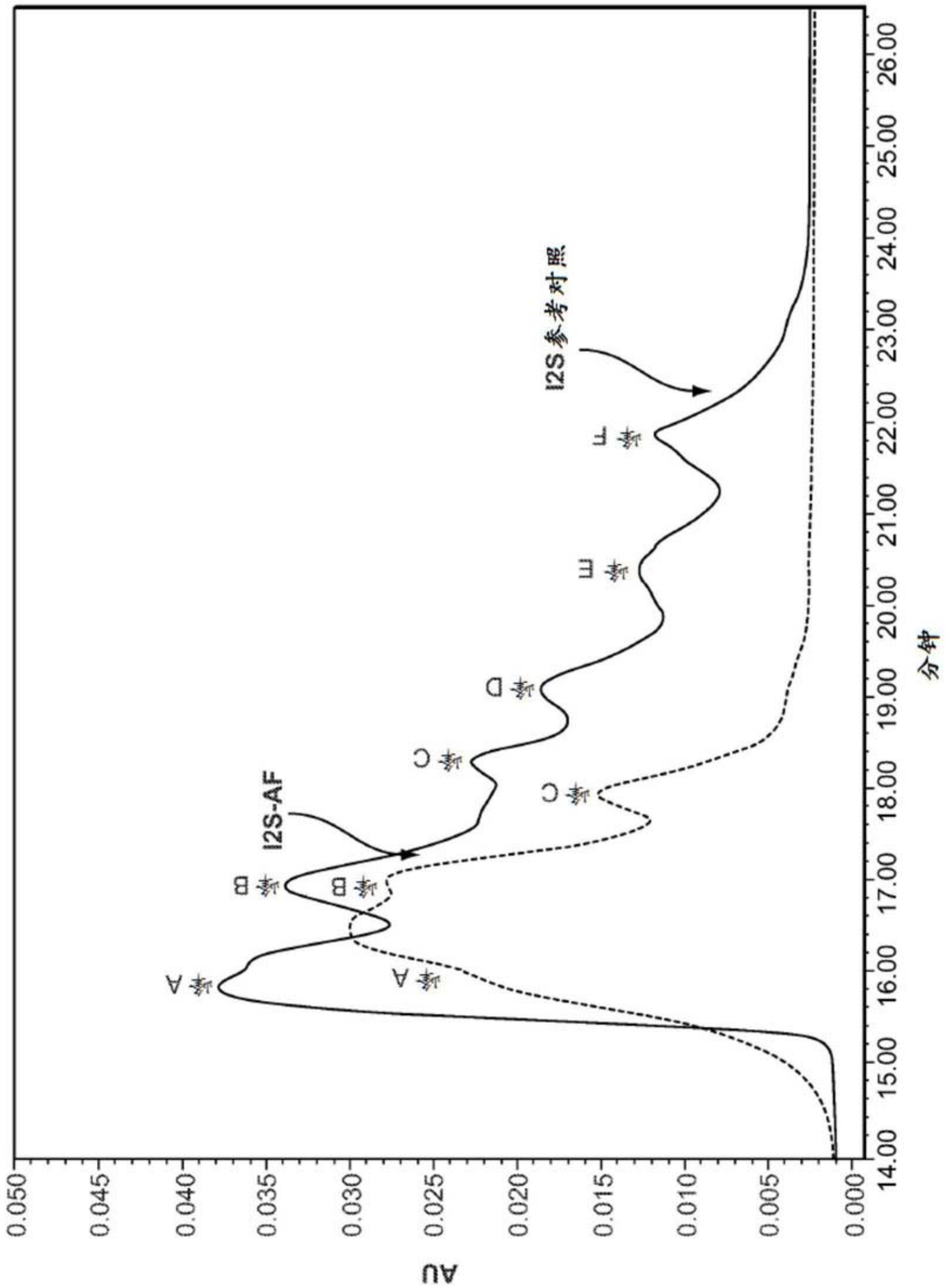

AU
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
22.00
23.00
24.00
25.00
26.00
分钟
峰 A
峰 B
峰 C
峰 D
峰 E
峰 F
峰 A
峰 B
峰 C
I2S-AF
I2S参考对照

图 11

Abstract

The present invention provides, among other things, improved methods for purifying 12 S protein produced recombinantly for enzyme replacement therapy. The present invention is, in part, based on the surprising discovery that recombinant I2S protein can be purified from unprocessed biological materials, such as, I2S-containing cell culture medium, using a process involving as few as four chromatography columns.

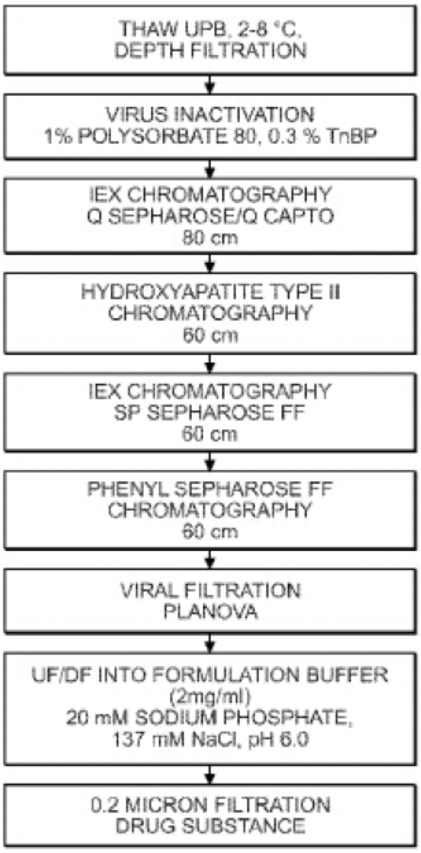


FIG. 1