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

[0006] The present disclosure provides, among other things, improved methods for purifying I2S protein produced recombinantly for enzyme replacement therapy. The present disclosure 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 disclosure conforms with the marketing purity requirements in the US and many other countries. In addition, the recombinant I2S enzyme purified according to the present disclosure 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 disclosure provides an effective, cheaper, and faster process for purifying recombinant I2S protein. The present disclosure is particularly useful for purifying recombinant I2S protein produced in serum-free medium.

[0006a] In one aspect, the present disclosure provides the use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1, wherein the purified recombinant I2S comprises at least 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly), wherein the purified recombinant I2S contains less than 150 ng/mg Host Cell Protein (HCP).

[0006b] In another aspect, the present disclosure provides the use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1, wherein the purified recombinant I2S comprises at least 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly), and wherein the purified recombinant I2S contains at least 10% bis-phosphorylated oligosaccharides per molecule.

[0006c] In another aspect, the present disclosure provides the use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) wherein the purified recombinant I2S comprises at least 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly), and wherein the purified recombinant I2S protein has specific activity of at least 40 U/mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

[0006d] In another aspect, the present disclosure provides the use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1, wherein the purified recombinant I2S comprises at least 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly), and wherein the purified recombinant I2S protein has specific activity of at least 20 U/mg as determined by an *in vitro* 4-MUF-SO_4 to 4-MUF conversion assay.

[0006e] In another aspect, the present disclosure provides the use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1, wherein the purified recombinant I2S (i) comprises at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly), and (ii) contains on average at least 16 sialic acids per molecule.

[0007] Thus, in one example, the present disclosure 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 examples, an inventive method according to the present disclosure involves less than 6 (e.g., less than 5, less than 4, or less than 3) chromatography steps. In some examples, an inventive method

according to the present disclosure involves 2, 3, 4 or 5 chromatography steps. In some examples, an inventive method according to the present disclosure involves 4 chromatography steps. In some examples, the purified recombinant I2S protein according to the present disclosure 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 examples, a suitable anion-exchange chromatography is Q chromatography. In some examples, a suitable cation-exchange chromatography is SP chromatography. In some examples, a suitable mixed-mode chromatography is hydroxyapatite (HA) chromatography. In some examples, 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 examples, a method according to the present disclosure 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 examples, 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 examples, the pH is adjusted using 1M sodium acetate. In some examples, the conductivity is adjusted using 5 M sodium chloride. In some examples, 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 examples, the anion-exchange chromatography column is eluted using a elution buffer comprising a linear salt (e.g., NaCl) gradient. In some examples, 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 examples, 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 examples, 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 examples, 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 examples, the conductivity is adjusted by dialfiltration. In some examples, 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 examples, 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 examples, a suitable pH is about 5.0-6.5 (e.g., about 5.0, 5.5, 6.0, or 6.5).

[0012] In some examples, 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 examples, 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 examples, 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 examples, 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

examples, elution from a mixed-mode chromatography column (e.g., HA column) is achieved with a gradient phosphate buffer. In some examples, 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 examples, elution from an HA column is achieved by stepwise increasing the phosphate concentration in the elution buffer. In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, an inventive method according to the present disclosure includes a step of viral inactivation before loading the impure preparation onto the first chromatography column. In some examples, the step of viral inactivation includes adding a detergent to the impure preparation. In some examples, an inventive method according to the disclosure further includes a step of viral removal after the last Chromatography column. In some examples, a method of the disclosure further includes a step of ultrafiltration and/or dialfiltration. In some examples, the step of ultrafiltration and/or dialfiltration includes exchanging the purified recombinant I2S protein into a drug formulation buffer.

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

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

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

[0019] In some examples, a purified recombinant I2S protein according to the present disclosure 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 examples, a purified recombinant I2S protein according to

the present disclosure contains, on average, 16, 17, 18, 19, 20, 21, or 22 sialic acids per molecule.

[0020] In some examples, a purified recombinant I2S protein according to the present disclosure 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 examples, a purified recombinant I2S protein according to the present disclosure 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 examples, a purified recombinant I2S protein according to the present disclosure 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 examples, a purified recombinant I2S protein according to the present disclosure 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 examples, a purified recombinant I2S protein according to the present disclosure 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 examples, the glycan map is generated following a neuraminidase digestion. In other examples, the glycan map is generated following an alkaline phosphatase digestion.

[0023] Among other things, the present disclosure provides purified recombinant I2S protein as described herein, and pharmaceutical compositions or formulation containing the same. In some examples, a formulation is formulated for intravenous, subcutaneous and/or intrathecal administration. The present disclosure 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 disclosure are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating examples of the present disclosure, is given by way of illustration only, not limitation. Various changes and modifications within the scope of the disclosure 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.

What is claimed is:

1. Use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1, wherein the purified recombinant I2S comprises at least 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly), wherein the purified recombinant I2S contains less than 150 ng/mg Host Cell Protein (HCP).

2. The use of claim 1, wherein the purified recombinant I2S comprises at least 75% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

3. The use of claim 1, wherein the purified recombinant I2S comprises at least 85% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

4. Use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1,

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

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

5. The use of claim 4, wherein the purified recombinant I2S contains at least 50% bis-phosphorylated oligosaccharides per molecule.

6. Use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S)

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

wherein the purified recombinant I2S protein has specific activity of at least 40 U/mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

7. The use of claim 6, wherein the purified recombinant I2S protein has specific activity of at least 50 U/mg as determined by an *in vitro* sulfate release activity assay using heparin disaccharide as substrate.

8. The use of claim 6, 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.

9. Use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1,

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

wherein the purified recombinant I2S protein has specific activity of at least 20 U/mg as determined by an *in vitro* 4-MUF-SO_4 to 4-MUF conversion assay.

10. The use of claim 9, wherein the purified recombinant I2S protein has a specific activity of at least 30 U/mg as determined by an *in vitro* 4-MUF-SO_4 to 4-MUF conversion assay.

11. The use of claim 10, wherein the purified recombinant I2S protein has a specific activity of at least 40 U/mg as determined by an *in vitro* 4-MUF-SO_4 to 4-MUF conversion assay.

12. The use of claim 10, wherein the purified recombinant I2S protein has a specific activity of at least 50 U/mg as determined by an *in vitro* 4-MUF-SO_4 to 4-MUF conversion assay.

13. The use of claim 10, wherein the purified recombinant I2S protein has a specific activity of at least 60 U/mg as determined by an *in vitro* 4-MUF-SO_4 to 4-MUF conversion assay.

14. Use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1, wherein the purified recombinant I2S

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

(ii) contains on average at least 16 sialic acids per molecule.

15. Use of a composition for the preparation of a medicament for the treatment of Hunter Syndrome wherein the composition comprises purified recombinant iduronate-2-sulfatase (I2S) having the amino acid sequence of SEQ ID NO:1,

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

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.

16. The use of claim 15, wherein the purified recombinant I2S protein contains less than 100 ng/mg HCP.

17. The use of claim 15, wherein the purified recombinant I2S protein contains less than 80 ng/mg HCP.

18. The use of any one of claims 1-17, wherein the treatment comprises that the medicament is to be intrathecally administered.