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

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

[0028a] In another aspect, the present invention provides a preparation of recombinant iduronate-2-sulfatase (I2S) protein, said recombinant I2S protein having an amino acid sequence at least 70% identical to SEQ ID NO:1 and comprising at least about 70% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Cα-formylglycine (FGly).

[0028b] In another aspect, the present invention provides the use of the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating Hunter syndrome.

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

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

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

BRIEF DESCRIPTION OF THE DRAWINGS

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

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

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

We claim:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

CLAIMS

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

2. The method of claim 1, wherein:
(a) said culturing of mammalian cells is under conditions such that the cells, on average, produce the recombinant I2S protein at a specific productivity rate of greater than about 15 picogram/cell/day or greater than about 30 picogram/cell/day, and further wherein the produced recombinant I2S protein, on average, comprises at least about 60%, at least about 70%, at least about 80% or at least about 97% conversion of the cysteine residue corresponding to Cys59 of human I2S protein to C_α-formylglycine, and/or
(b) the culturing step comprises a perfusion process, optionally wherein the perfusion process has (i) a perfusion rate ranging from about 0.5-2 volume of fresh medium/working volume of reactor/day (WD), or (ii) a cell specific perfusion rate ranging from about 0.05-5 nanoliter per cell per day (nL/cell/day), and/or
(c) the cells produce the recombinant I2S protein at an average harvest titer of at least 6 mg per liter per day.

3. The method of claim 1 or claim 2, wherein:
(a) the mammalian cells are human cells or CHO cells, and/or
(b) the large-scale culture vessel is a bioreactor, optionally wherein the bioreactor is at a scale of or greater than 10L, 200L, 500L, 1000L, 1500L, or 2000L, and/or
(c) the medium lacks animal-derived components, and/or
(d) the medium is chemically-defined medium, and/or
(e) the medium is protein free-.

4. The method of any one of claims 1-3, wherein the medium comprises at least one redox-modulator.

5. The method of claim 4, wherein:
(a) the at least one redox-modulator is selected from the group consisting of glutathione, glucose-6-phosphate, carnosine, carnosol, sulforaphane, tocopherol, ascorbate, dehydroascorbate, selenium, 2-mercaptoenthanol, N-acetylcysteine, cysteine, riboflavin,

niacin, folate, flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADP), optionally:

(i) wherein the at least one redox-modulator comprises cysteine, and optionally wherein the cysteine is at a concentration ranging from about 0.1 mg/L to about 65 mg/L, and/or

(ii) wherein the at least one redox-modulator comprises 2-mercaptoenthanol, optionally wherein the 2-mercaptoenthanol is at a concentration ranging from about 0.001 mM to about 0.01 mM, and/or

(iii) wherein the at least one redox-modulator comprises N-acetylcysteine, optionally wherein the N-acetylcysteine is at a concentration ranging from about 3 mM to about 9 mM, and/or

(b) the medium comprises at least one growth-modulator, optionally:

(i) wherein the at least one growth-modulator comprises hypoxanthine, optionally wherein the hypoxanthine is at a concentration ranging from about 0.1 mM to about 10 mM, and/or

(b) wherein the at least one growth-modulator comprises thymidine, optionally wherein the thymidine is at a concentration ranging from about 1 mM to about 100 mM.

6. The method of any one of claims 1-5, wherein the medium has a pH ranging from about 6.8 - 7.5, optionally wherein the medium has a pH ranging from about 6.9-7.3.

7. The method of any one of claims 1-6, wherein:

(a) the culturing step comprises a growth phase and a production phase, and/or

(b) the mammalian cells are cultured at a temperature ranging from 30-37 °C, optionally wherein:

(i) the mammalian cells are cultured at different temperatures during the growth phase and the production phase, and/or

(ii) the medium for the growth phase and the production phase has different pH, and/or

(iii) the mammalian cells are maintained at a viable cell density ranging from about 1.0-50 X 10^6 viable cells/mL during the production phase, and/or

(iv) the production phase is lasted for about 5-90 days.

8. The method of any one of claims 1-7, wherein the method further comprises a step of harvesting the recombinant I2S protein.

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9. The method of any one of claims 1-8, wherein:
(a) the recombinant I2S protein comprises an amino acid sequence at least 70% identical to SEQ ID NO:1, and optionally wherein the recombinant I2S protein comprises an amino acid sequence identical to SEQ ID NO:1, and/or
(b) the FGE comprises an amino acid sequence at least 70% identical to SEQ ID NO:5, optionally wherein the FGE comprises an amino acid sequence identical to SEQ ID NO:5, and/or
(c) the cells comprise one or more exogenous nucleic acids encoding the recombinant I2S protein and/or the FGE, optionally wherein:
    (i) the one or more exogenous nucleic acids are integrated in the genome of the cells, or
    (ii) the one or more exogenous nucleic acids are present on one or more extra-chromosomal constructs, and/or
(d) the cells over-express the recombinant I2S protein, and/or
(e) the cells over-express the FGE.

10. A recombinant iduronate-2-sulfatase (I2S) protein produced using a method of any one of claims 1-9.

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

12. The preparation of claim 11, wherein:
(a) the recombinant I2S protein comprises at least about 80%, at least about 90%, at least about 95% or at least about 97% conversion of the cysteine residue corresponding to Cys59 of SEQ ID NO:1 to Fgly, and/or
(b) the recombinant I2S protein has specific activity of at least 40 U/mg, at least 60 U/mg or at least 80 U/mg as determined by an in vitro sulfate release activity assay using heparin disaccharide as substrate.

13. The preparation of claim 11 or claim 12, wherein the recombinant I2S protein comprises an amino acid sequence at least 70% identical to SEQ ID NO:1, optionally wherein the recombinant I2S protein comprises an amino acid sequence identical to SEQ ID NO:1.

14. A pharmaceutical composition comprising a recombinant I2S protein of claim 12 or claim 13 and a pharmaceutically acceptable carrier.

15. The pharmaceutical composition of claim 14 for use in a method of treating Hunter syndrome.

16. Use of the pharmaceutical composition of claim 14 in the manufacture of a medicament for treating Hunter syndrome.