[0007] Accordingly, the therapeutic effectiveness of a lysosomal sulfatase enzyme preparation depends on the level of mannose-6-phosphate, and on the presence of active enzyme, in that preparation.

[0008] Thus, there exists a need in the art for an efficient and productive system for the large-scale manufacture of therapeutically effective, active highly phosphorylated lysosomal sulfatase enzymes for management of lysosomal storage disorders caused by or associated with a deficiency of such lysosomal sulfatase enzymes.

SUMMARY OF INVENTION

[0009] The present ~~invention~~disclosure relates to the discovery that when a CHO-K1 cell line derivative (designated G71) that is defective in endosomal acidification is engineered to express recombinant human sulfatase modifying factor 1 (SUMF1), the modified G71 cells produce high yields of active highly phosphorylated recombinant lysosomal sulfatase enzymes in part by preventing loss of material to the lysosomal compartment of the manufacturing cell line. In one ~~embodiment~~example, the ~~invention~~present disclosure provides an END3 complementation group cell line that co-expresses recombinant human SUMF1 and recombinant human N-acetylgalactosamine-6-sulfatase (GALNS), resulting in high yields of active highly phosphorylated enzyme. Exemplary cell lines are G71, G71S, and derivatives thereof, which retain the desired property of G71, *i.e.*, the ability to produce high yields of activate highly phosphorylated recombinant lysosomal sulfatase enzymes. This application of an END3 complementation group modified CHO-K1 cell line co-expressing recombinant human SUMF1 and a recombinant lysosomal sulfatase enzyme would be especially useful for the manufacture of active highly phosphorylated lysosomal sulfatase enzymes to be used for management of lysosomal storage diseases by enzyme replacement therapy (ERT).

[0010] Disclosed is a~~In a first aspect, the present invention features a novel~~ method of producing active highly phosphorylated recombinant human lysosomal sulfatase enzymes or biologically active fragments, mutants, variants or derivatives thereof in an END3 complementation group CHO cell or derivative thereof in amounts that enable their therapeutic use. In ~~a broad embodiment~~one example, the method comprises the steps of: (a) culturing a CHO-derived END3 complementation group cell or derivative thereof; (b) preparing a first mammalian expression vector capable of expressing the active highly phosphorylated recombinant human lysosomal sulfatase enzyme or biologically active fragment, mutant, variant or derivative thereof in the CHO-derived END3 complementation group cell or derivative thereof; (c) preparing a second

mammalian expression vector capable of expressing recombinant human sulfatase modifying factor 1 (SUMF1) or biologically active fragment, mutant, variant or derivative thereof in the CHO-derived END3 complementation group cell or derivative thereof; (d) transfecting the CHO-derived END3 complementation group cell or derivative thereof with the first and second expression vectors; (e) selecting and cloning of a transfectant of a CHO-derived END3 complementation group cell or derivative thereof that expresses the active highly phosphorylated recombinant human lysosomal sulfatase enzyme or biologically active fragment, mutant, variant or derivative thereof; and (f) optimizing a cell culture process method for manufacturing the highly phosphorylated recombinant human lysosomal sulfatase enzyme or biologically active fragment, mutant, variant or derivative thereof. The recombinant human lysosomal sulfatase enzyme is selected from the group consisting of arylsulfatase A (ARSA), arylsulfatase B (ARSB), iduronate-2-sulfatase (IDS), sulfamidase/heparin-N-sulfatase (SGSH), N-acetylglucosamine-sulfatase (G6S) and N-acetylgalactosamine-6-sulfatase (GALNS).

[0011] The method involves the steps of transfecting a cDNA encoding all or part of the lysosomal sulfatase enzyme and a cDNA encoding all or part of the human SUMF1 into a CHO-derived END3 complementation group cell or derivative thereof. In some ~~embodiments~~examples, the first and second expression vectors, which are capable of expressing the encoding the active highly phosphorylated recombinant human lysosomal sulfatase enzyme and human SUMF1, respectively, are transfected simultaneously into the CHO-derived END3 complementation group cell or derivative thereof. In some ~~embodiments~~examples, the first and second expression vectors are transfected into the CHO-derived END3 complementation group cell or derivative thereof sequentially. In some ~~embodiments~~examples, a cDNA encoding for a full-length human lysosomal sulfatase enzyme is used, whereas in other ~~embodiments~~examples a cDNA encoding for a biologically active fragment, mutant, variant or derivative thereof is used. In some ~~embodiments~~examples, a cDNA encoding for a full-length human SUMF1 is used, whereas in other ~~embodiments~~examples a cDNA encoding for a biologically active fragment, mutant, variant or derivative thereof is used. In some ~~embodiments~~examples, multiple expression vectors are used to transfer the human lysosomal sulfatase enzyme and human SUMF1 cDNAs simultaneously or sequentially into the CHO-derived END3 complementation group cell or derivative thereof. In some ~~embodiments~~examples, a single expression vector is used to transfer the human lysosomal sulfatase enzyme and human SUMF1 cDNAs simultaneously into the CHO-derived END3 complementation group cell or derivative thereof. In ~~one a preferred~~

~~embodiment~~example, the CHO-derived END3 complementation group cell or derivative thereof is a G71 cell line, a G71S cell line, or a G71 or G71S derivative.

[0012] In ~~a preferred embodiment~~one example, the method comprises producing an active highly phosphorylated recombinant human lysosomal sulfatase enzyme, *e.g.*, arylsulfatase A (ARSA), arylsulfatase B (ARSB), iduronate-2-sulfatase (IDS), sulfamidase/heparin-N-sulfatase (SGSH), N-acetylglucosamine-sulfatase (G6S) or N-acetylgalactosamine-6-sulfatase (GALNS), from an END3 complementation group CHO cell line or derivative thereof. In ~~a particularly preferred embodiment~~another example, the method comprises producing active highly phosphorylated recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) from an END3 complementation group CHO cell line or derivative thereof. An END3 complementation group cell line is any modified CHO cell line that retains the properties of an END3 complementation group cell line, such as defective endosomal acidification. In ~~a preferred embodiment~~another example, the CHO-derived END3 complementation group cell or derivative thereof is a G71 cell line, a G71S cell line, or a G71 or G71S derivative.

[0013] Also disclosed is~~In a second aspect, the present invention provides~~ an endosomal acidification-deficient mammalian cell line characterized by its ability to produce active highly phosphorylated recombinant human lysosomal sulfatase enzymes in amounts that enable use of the lysosomal sulfatase enzyme therapeutically. In ~~preferred embodiments~~one example, the ~~invention~~present disclosure provides CHO-K1-derived END3 complementation group cell lines, designated G71, G71S, or derivatives thereof, which are capable of producing high yields of active highly phosphorylated recombinant human lysosomal sulfatase enzymes, thereby enabling the large scale production of such therapeutic lysosomal sulfatase enzymes. In ~~more preferred embodiments~~one example, the cell line expresses and secretes a recombinant human lysosomal sulfatase enzyme in amounts of at least about 0.5, preferably at least about 0.75, more preferably at least about 1.0, and even more preferably at least about 1.25 picograms/cell/day.

[0014] An END3 complementation group cell line is any modified CHO cell line that retains the properties of an END3 complementation group cell line, such as defective endosomal acidification. In one ~~embodiment~~example, the END3 complementation group CHO cell line is derived from G71 or a derivative thereof and comprises (a) an expression vector for recombinant human sulfatase modifying factor 1 (SUMF1) and (b) an expression vector for a recombinant human lysosomal sulfatase enzyme, wherein the recombinant human lysosomal sulfatase enzyme is selected from the group consisting of arylsulfatase A (ARSA), arylsulfatase B (ARSB), iduronate-2-sulfatase (IDS), sulfamidase/heparin-N-sulfatase

(SGSH), N-acetylglucosamine-sulfatase (G6S) and N-acetylgalactosamine-6-sulfatase (GALNS). In ~~a preferred embodiment~~one example, the END3 complementation group CHO cell line comprises the expression vector for recombinant human N-acetylgalactosamine-6-sulfatase (GALNS). In ~~a more preferred embodiment~~another example, the END3 complementation group CHO cell line expresses and secretes recombinant human GALNS. In another ~~preferred embodiment~~example, the END3 complementation group CHO cell line is selected from the group consisting of clone 4, clone 5, clone C6, clone C2, clone C5, clone C7, clone C10, clone C11 and clone C30. In ~~a more preferred embodiment~~another example, the END3 complementation group CHO cell line is clone C2. In another ~~preferred embodiment~~example, the END3 complementation group CHO cell line is adapted to growth in suspension.

[0015] Also disclosed are~~In a third aspect, the invention provides~~ recombinant human lysosomal sulfatase enzymes produced in accordance with the methods of the present ~~invention~~disclosure and thereby present in amounts that enable using the lysosomal sulfatase enzymes therapeutically. The lysosomal sulfatase enzymes may be full-length proteins, or fragments, mutants, variants or derivatives thereof. In some ~~embodiments~~examples, the lysosomal sulfatase enzyme or fragment, mutant, variant or derivative thereof according to the ~~invention~~present disclosure may be modified as desired to enhance its stability or pharmacokinetic properties (*e.g.*, PEGylation, mutagenesis, fusion, conjugation). In ~~preferred embodiments~~one example, the enzyme is a human lysosomal sulfatase enzyme, a fragment of the human lysosomal sulfatase enzyme having a biological activity of a native sulfatase enzyme, or a polypeptide that has substantial amino acid sequence homology with the human lysosomal sulfatase enzyme. In some ~~embodiments~~examples, the lysosomal sulfatase enzyme is a protein of human or mammalian sequence, origin or derivation. In other ~~embodiments~~examples, the lysosomal sulfatase enzyme is such that its deficiency causes a human disease, such as Metachromic Leukodystrophy or MLD (*i.e.*, arylsulfatase A (ARSA)), Maroteaux-Lamy syndrome or MPS VI (*i.e.*, arylsulfatase B (ARSB)), Hunter syndrome or MPS II (*i.e.*, iduronate-2-sulfatase (IDS)), Sanfilippo A syndrome or MPS IIIa (*i.e.*, sulfamidase/heparin-N-sulfatase (SGSH)), Sanfilippo D syndrome or MPS IIId (*i.e.*, N-acetylglucosamine-sulfatase (G6S)) and Morquio A syndrome or MPS IVa (*i.e.*, N-acetylgalactosamine-6-sulfatase (GALNS)). In ~~a particularly preferred embodiment~~one example, the lysosomal sulfatase enzyme is such that its deficiency causes Morquio A syndrome or MPS IVa (*i.e.*, N-acetylgalactosamine-6-sulfatase (GALNS)). In another ~~particularly preferred embodiment~~example, the lysosomal sulfatase enzyme is such that its deficiency is associated with a

human disease, such as Multiple Sulfatase Deficiency or MSD (*i.e.*, N-acetylgalactosamine-6-sulfatase (GALNS)).

[0015a] In one aspect, there is provided a composition purified as defined above, said composition comprising a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) enzyme, said GALNS enzyme comprising an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, wherein said GALNS enzyme:

(i) has a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions,

(ii) has at least about 50% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and

(iii) has between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain,

and wherein at least 98% of said GALNS enzyme is in the precursor form as determined by SDS-CGE.

[0016] The lysosomal sulfatase enzyme can also be of human or mammalian sequence origin or derivation. In yet other ~~embodiments of the invention~~examples, in each of its ~~aspects~~examples, the lysosomal sulfatase enzyme is identical in amino acid sequence to the corresponding portion of a human or mammalian lysosomal sulfatase enzyme amino acid sequence. In other ~~embodiments~~examples, the polypeptide moiety is the native lysosomal sulfatase enzyme from the human or mammal. In other ~~embodiments~~examples, the lysosomal sulfatase enzyme polypeptide is substantially homologous (*i.e.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical in amino acid sequence) over a length of at least about 25, 50, 100, 150, or 200 amino acids, or the entire length of the polypeptide, to the native lysosomal sulfatase enzyme amino acid sequence of the human or mammalian enzyme. In some ~~embodiments~~examples, the lysosomal sulfatase enzyme is human N-acetylgalactosamine-6-sulfatase (GALNS). The amino acid sequence of human GALNS is set forth in SEQ ID NO: 4, of which amino acids 27 to 522 correspond to the secreted precursor protein. In some ~~embodiments~~examples, this GALNS enzyme comprises or consists of an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to amino acids 27 to 522 of SEQ ID NO: 4, or a sequence identical to amino acids 27 to 522 of SEQ ID NO: 4. The GALNS enzyme preferably retains the catalytic site amino acids corresponding to the Cys at position 53 of the secreted precursor protein (amino acid 79 of SEQ ID NO: 4), which is capable of being

converted to C_{α}-formylglycine. The GALNS enzyme may also retain other amino acids in the active site cavity, including at least 1, 2, 3, 4, 5, 6, 7, 8 or all of the charged amino acids: Asp288, Asn289, Asp39, Asp54, His236, Lys140, His142, Lys310 and the α-helix: Arg83. Sukegawa, *Human Molecular Genetics*, 2000, Vol. 9, No. 9 1283-1290, incorporated herein by reference in its entirety, describes additional mutations that decrease GALNS activity in patients and correlates the severity of various mutations to their respective 3-dimensional location within the enzyme. In other ~~embodiments~~examples, the subject to which the lysosomal sulfatase enzyme is to be administered is human.

[0017] In ~~preferred embodiments~~one example, the lysosomal sulfatase enzyme is a highly phosphorylated recombinant human lysosomal sulfatase enzyme produced by an endosomal acidification-deficient cell line, *e.g.*, a CHO-derived END3 complementation group cell line. An END3 complementation group cell line is any modified CHO cell line that retains the properties of an END3 complementation group cell line, such as defective endosomal

acidification. In ~~a preferred embodiment~~one example, the CHO-derived END3 complementation group cell or derivative thereof is a G71 cell line, a G71S cell line, or a G71 or G71S derivative. Alternatively, the lysosomal sulfatase enzyme may be produced by any host cell, *e.g.*, any CHO cell or CHO cell-derived line, cultured under conditions that permit expression and secretion of highly phosphorylated recombinant lysosomal sulfatase enzyme at relatively high yield, *e.g.*, in amounts of at least about 0.5, at least about 0.75, at least about 1.0, or at least about 1.25 picograms/cell/day.

[0018] In ~~more preferred embodiments~~one example, the recombinant human lysosomal sulfatase enzyme has a high level of phosphorylated oligosaccharides (*i.e.*, greater than about 0.25, preferably greater than 0.5, and more preferably greater than about 0.75 bis-phosphorylated oligomannose chains per protein chain).

[0019] In some ~~embodiments~~examples, the ~~invention~~present disclosure provides a recombinant human lysosomal sulfatase enzyme, *e.g.*, GALNS, with a specified high level of phosphorylated oligosaccharides. For example, the lysosomal sulfatase enzyme has from 0.5 to 1.0 bis-phosphorylated oligomannose chains per monomeric protein chain, or from 0.5 to 0.9 bis-phosphorylated oligomannose chains per monomeric protein chain, or from 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain, or from 0.5 to 0.75 bis-phosphorylated oligomannose chains per monomeric protein chain, or from 0.54 to 0.75 bis-phosphorylated oligomannose chains per monomeric protein chain. Other similar ranges are contemplated, *e.g.*, at least 0.4, 0.45, 0.5, 0.55, 0.6 or 0.65 bis-phosphorylated oligomannose chains per monomeric protein chain, up to 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.98, or 1.0 bis-phosphorylated oligomannose chains per monomeric protein chain, or any combination of any of these numbers. In ~~preferred embodiments~~one example, the enzyme is a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS), *e.g.*, of SEQ ID NO: 4.

[0020] In some ~~embodiments~~examples, the recombinant human lysosomal sulfatase enzyme has a high percentage (*i.e.*, at least about 50%, 55%, 60%, or 65%, preferably at least about 70%, 75%, 80%, 85%, 90%, or 95%) of conversion of the active site cysteine residue to C_α-formylglycine (FGly). In preferred ~~embodiments~~examples, the enzyme is an active recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) and the active site cysteine residue is the Cys at position 53 (position 79 of SEQ ID NO: 4).

[0021] In particular ~~embodiments~~examples, the recombinant human lysosomal sulfatase enzyme has a high level of phosphorylated oligosaccharides, *e.g.*, any of the ranges or levels of bis-

phosphorylated oligomannose chains per monomeric protein chain described herein, together with a high percentage of conversion of the active site cysteine residue to C_α-formylglycine (FGly), *e.g.*, any of the percentages described herein. In preferred ~~embodiments~~examples, the enzyme is an active highly phosphorylated recombinant human N-acetylgalactosamine-6-sulfatase (GALNS), *e.g.*, of SEQ ID NO: 4.

[0022] In any of the preceding ~~embodiments~~examples, at least 99.5%, at least 99%, at least 98.5%, at least 98%, at least 97%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, or at least 65% of the recombinant human lysosomal sulfatase enzyme, *e.g.*, GALNS (SEQ ID NO: 4), is in the precursor form as determined by Coomassie Blue staining when subjected to SDS-PAGE under reducing conditions or by SDS-capillary gel electrophoresis (SDS-CGE).

[0023] In addition, the lysosomal sulfatase enzyme, *e.g.*, GALNS (SEQ ID NO: 4), optionally also exhibits a specific activity that is at least about 30% (*e.g.*, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 2-fold, 2.5-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold or 50-fold) greater than the specific activity of a control lysosomal sulfatase enzyme of the same amino acid sequence that has been produced in host cells (*e.g.*, CHO cells or CHO-derived cells) that do not express recombinant human SUMF1.

[0024] In any of the preceding ~~embodiments~~examples, the lysosomal sulfatase enzyme described, *e.g.*, GALNS (SEQ ID NO: 4), exhibits a specific uptake (Kuptake) into fibroblasts that is about 0.1 to 10 nM, or about 0.1 to 7 nM, or about 0.5 to 5 nM, or about 1 to 5 nM, or about 1 to 3.5 nM, about 1 nM, about 1.5 nM, about 2 nM, about 2.5 nM, about 3 nM or about 3.5 nM, or any combination of any of these numbers.

[0025] In any of the preceding ~~embodiments~~examples, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80% of the recombinant human lysosomal sulfatase enzyme, *e.g.*, GALNS (SEQ ID NO: 4), binds a mannose-6-phosphate receptor column.

[0025a] In one aspect, there is provided a formulation comprising

(a) recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) enzyme purified as defined herein, said GALNS enzyme comprising an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, and

(i) having a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions,

(ii) having at least about 50% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and

(iii) having between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain,

wherein at least 98% of said GALNS enzyme is in the precursor form as determined by SDS-CGE, and

(b) one or more pharmaceutically acceptable carriers, diluents or excipients comprising:

(i) an amount of phosphate buffer effective to reduce dephosphorylation of said GALNS enzyme; and

(ii) a stabilizing amount of one or more stabilizers selected from the group consisting of amino acid salts, amino acid buffers, surfactants and polyols;

wherein said formulation is at a pH of about 5.0-5.8.

[0026] According to this aspect, purified preparations of any of these ~~embodiments~~examples of the lysosomal sulfatase enzyme are provided in which the lysosomal sulfatase enzyme component, *e.g.*, GALNS (SEQ ID NO: 4) has a purity of at least about 90%, 95%, 97%, 98%, or 99% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions or by another method of determining purity (*e.g.*, SDS-PAGE under

reducing or non-reducing conditions followed by staining with Coomassie Blue or silver, or chromatographic separation by HPLC, including C4 reverse phase (RP) or C3 RP), or size exclusion chromatography (SEC)). In some ~~embodiments~~examples, a significant amount of the lysosomal sulfatase enzyme component of the purified preparation is in the secreted precursor form (*e.g.*, at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 98.5%, 99% or 99.5% precursor), as determined by Coomassie Blue staining when subjected to SDS-PAGE under reducing conditions or by another method of detecting precursor (*e.g.*, SDS-PAGE under reducing conditions with Coomassie Blue or silver staining, or chromatographic separation by HPLC (*e.g.*, C4 reverse phase (RP), C3 RP) or size exclusion chromatography (SEC), or a combination of electrophoretic separation and chromatographic separation, *e.g.*, SDS-PAGE followed by capillary gel electrophoresis (SDS-CGE)).

[0027] In particular ~~embodiments~~examples, the lysosomal sulfatase enzyme component of the purified preparation has a high level of phosphorylated oligosaccharides, *e.g.*, any of the ranges or levels of bis-phosphorylated oligomannose chains per monomeric protein chain described herein, together with a high percentage of conversion of the active site cysteine residue to C_α-formylglycine (FGly), *e.g.*, any of the percentages described herein. In more particular ~~embodiments~~examples, the purified preparation has a Kuptake as described herein.

[0028] In related aspects, the ~~invention~~present disclosure provides sterile compositions containing any of the lysosomal sulfatase enzyme or purified preparations described herein, together with a sterile pharmaceutically acceptable diluent, carrier and/or excipient. Such sterile compositions may take the form of solutions or lyophilized powder, optionally in vials, that may be reconstituted by the addition of sterile diluent.

[0029] Also disclosed is~~In a fourth aspect, the invention provides~~ a method to purify recombinant human lysosomal sulfatase enzymes produced by the methods of the present ~~invention~~disclosure. In a preferred ~~embodiment~~example, lysosomal sulfatase enzymes are purified using a two-column process (dye-ligand chromatography, *e.g.*, Blue-Sepharose, and cation exchange chromatography, *e.g.*, SE Hi-Cap) comprising at least five purification steps: (1) filtering the harvest, *i.e.*, culture medium from an END3 complementation group CHO cell line or derivative thereof that expresses human sulfatase modifying factor 1 (SUMF1) and the recombinant human lysosomal sulfatase enzyme; (2) pH adjusting the filtered harvest to pH 4.5 (to induce precipitation of contaminating proteins); (3) loading the pH-adjusted filtered harvest onto a dye-ligand column, *e.g.*, Blue-Sepharose column, washing the column and eluting the lysosomal sulfatase enzyme from the column; (4) loading the eluate from the dye-ligand

column onto a cation exchange column, *e.g.*, SE Hi-Cap column, washing the column and eluting the lysosomal sulfatase enzyme from the column; and (5) ultrafiltrating and diafiltrating the eluate from the cation exchange. Optionally, the filtered harvest in step (1) is concentrated 10-20 fold by ultrafiltration before adjusting the pH. Optionally, the ultrafiltrated and diafiltrated lysosomal sulfatase enzyme in step (5) is formulated in a formulation buffer. In ~~a particularly preferred embodiment~~one example, the lysosomal enzyme is a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS).

[0029a] In one aspect, there is provided a method of purifying a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) enzyme, said GALNS enzyme comprising an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, wherein said GALNS enzyme:

(i) has a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions,

(ii) has at least about 50% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and

(iii) has between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain,

and wherein at least 98% of said GALNS enzyme is in the precursor form as determined by SDS-CGE, comprising:

a) filtering a culture medium containing the GALNS enzyme secreted from a mammalian cell line that expresses human sulfatase modifying factor 1 (SUMF1) and the recombinant human GALNS enzyme,

ultrafiltering/diafiltering the filtered culture medium, whereby the ultrafiltered/diafiltered culture medium is concentrated about 20X, and

charcoal filtering the 20 X concentrated ultrafiltered/diafiltered culture medium;

b) loading the charcoal filtered 20X ultrafiltered/dialfiltered culture medium from step a) onto a Zn-chelating Sepharose FF capture column, washing the column under conditions such that the GALNS enzyme is retained on the column, and eluting the GALNS enzyme from the column;

c) optionally, filtering the eluate from the Zn-chelating Sepharose FF capture column in step b) through a filter to remove viruses;

d) adjusting the pH of the eluate from the Zn-chelating Sepharose FF capture column from step b) or the filtrate from step c) to about pH 4.5 ± 0.1, and filtering the pH 4.5-adjusted eluate from the Zn-chelating Sepharose FF capture column or filtrate;

e) loading the pH 4.5-adjusted eluate from the Zn-chelating FF capture column or filtrate from step d) onto a Fractogel EMD SE Hi-Cap cation exchange column, washing the column under conditions such that the GALNS enzyme is retained on the column, and eluting the GALNS enzyme from the column;

f) adjusting the pH of the eluate from the Fractogel EMD SE Hi-Cap cation exchange column from step e) to about pH 3.5 ± 0.1 for viral inactivation;

g) adjusting the pH 3.5 viral inactivated eluate from step f) to about pH 5.0, then loading the pH 5.0-adjusted viral inactivated eluate onto a ToyoPearl Butyl 650 M polishing column, washing the column under conditions such that the GALNS enzyme is retained on the column, and eluting the GALNS enzyme from the column;

h) buffer exchanging the eluate from the TovoPearl Butyl 650 M polishing column from step g) into a formulation comprising 20 mM NaOAc/HOAc, 50 mM NaH_2PO_4, 30 mM arginine HCl, 2% (w/v) sorbitol, pH 5.4, and adjusting the concentration of the GALNS enzyme in the formulation to about 3 mg/mL;

i) removing any residual virus and/or DNA by filtering the buffer exchanged formulation in step h) with a DV20 filter and a Mustang Q filter; and

j) adding polysorbate 20 (PS20) to the formulation in step i) to a final concentration of 0.01% (w/v).

[0030] In another ~~preferred embodiment~~example, lysosomal sulfatase enzymes are purified using a three-column process (capture chromatography, *e.g.*, cation exchange SE Hi-Cap; intermediate chromatography, *e.g.*, dye-ligand Capto Blue, Zinc Chelating Sepharose FF or Capto Adhere; and polishing chromatography, *e.g.*, ToyoPearl Butyl 650M, Phenyl Sepharose Hi-Sub or Phenyl Sepharose Low-Sub) comprising at least five purification steps: (1) ultrafiltering the harvest, *i.e.*, culture medium from an END3 complementation group CHO cell line or derivative thereof that expresses human sulfatase modifying factor 1 (SUMF1) and the recombinant human lysosomal sulfatase enzyme, by, *e.g.*, Sartocon Cassettes, (30 kDa, Hydrosart); (2) pH adjusting the filtered harvest to pH 4.5 (to induce precipitation of contaminating proteins); (3) loading the pH-adjusted filtered harvest onto a

capture column, *e.g.*, Fractogel EMD SE Hi-CAP (M) cation exchange, washing the column and eluting the lysosomal sulfatase enzyme from the column; (4) loading the eluate from the capture column onto an intermediate column, *e.g.*, dye-ligand Capto Blue, Zinc Chelating Sepharose FF or Capto Adhere, washing the column and eluting the lysosomal sulfatase enzyme from the column; and (5) loading the eluate on a polishing column, *e.g.*, ToyoPearl Butyl 650M, Phenyl Sepharose Hi-Sub or Phenyl Sepharose Low-Sub, washing the column and eluting the lysosomal sulfatase enzyme from the column. The eluted lysosomal sulfatase enzyme from step (5) is formulated in a formulation buffer. Optionally, the eluted lysosomal sulfatase enzyme from step (5) is ultrafiltrated and then formulated in a formulation buffer. Optionally, the lysosomal sulfatase enzyme from the column in step (4) is exposed to pH 3.5 for low pH viral inactivation prior to loading onto the polishing column in step (5). In ~~a particularly preferred embodiment~~one example, the lysosomal sulfatase enzyme is a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS).

[0031] In another ~~preferred embodiment~~example, lysosomal sulfatase enzymes are purified using a different three-column process (capture or immobilized metal affinity chromatography (IMAC), *e.g.*, dye-ligand Capto Blue, Zinc Chelating Sepharose FF or Capto Adhere;

intermediate chromatography, *e.g.*, Fractogel EMD SE Hi-Cap cation exchange; and polishing chromatography, *e.g.*, ToyoPearl Butyl 650M, Phenyl Sepharose Hi-Sub or Phenyl Sepharose Low-Sub) designed to reduce proteolytic digestion (*i.e.*, clipping) of the lysosomal sulfatase enzyme comprising at least six purification steps: (1) filtering the harvest, *i.e.*, culture medium from a mammalian cell line, *e.g.*, an END3 complementation group CHO cell line or derivative thereof, that expresses human sulfatase modifying factor 1 (SUMF1) and the recombinant human lysosomal sulfatase enzyme, ultrafiltering/diafiltering the filtered harvest by, *e.g.*, Sartocon Cassettes (30 kDa, Hydrosart), resulting in a concentrated filtered harvest, *e.g.*, 20X concentrated, and charcoal filtering the concentrated filtered harvest; (2) loading the charcoal filtered, concentrated harvest onto a capture or IMAC column, *e.g.*, dye-ligand Capto Blue, Zinc Chelating Sepharose FF or Capto Adhere, washing the capture column under conditions such that the lysosmal sulfatase enzyme is retained on the capture column, and eluting the lysosomal sulfatase enzyme from the capture column; (3) optionally, filtering the eluate from the capture column with a filter, *e.g.*, a Mustang Q filter, for removal of viruses; (4) adjusting the pH of the eluate or filtered eluate from the capture column to an acid pH, *e.g.*, pH 4.5 ± 0.1, then filtering the acid pH-adjusted eluate or filtered eluate from the capture column; (5) loading the filtered, acid pH-adjusted eluate or filtered eluate from the capture column onto an intermediate column, *e.g.*, Fractogel EMD SE Hi-CAP cation exchange column, washing the intermediate column under conditions such that the lysosomal sulfatase enzyme is retained on the intermediate column, and eluting the lysosomal sulfatase enzyme from the intermediate column; (6) adjusting the pH of the eluate from the intermediate column to low pH, *e.g.*, pH 3.5 ± 0.1, for viral inactivation; and (7) loading the low pH viral inactivated eluate from the intermediate cation exchange column onto a polishing column, *e.g.*, hydrophobic interaction chromatography (HIC) column, *e.g.*, ToyoPearl Butyl 650M, Phenyl Sepharose Hi-Sub or Phenyl Sepharose Low-Sub, washing the polishing column under conditions such that the lysosomal sulfatase enzyme is retained on the polishing column, and eluting the lysosomal sulfatase enzyme from the polishing column. In ~~a preferred embodiment~~one example, step (3) is included in the purification process. In another ~~preferred embodiment~~example, step (3) is omitted in the purification process. Optionally, (8) the eluted lysosomal sulfatase enzyme from step (7) is buffer exchanged into a formulation, *e.g.*, including but not limited to those formulations described herein, such as 20 mM NaOAc/HOAc, 50 mM NaH_2PO_4, 30 mM arginine HCl, 2% (w/v) sorbitol, pH 5.4, and the concentration of the eluted lysosomal sulfatase enzyme in the formulation is adjusted to an appropriate concentration, *e.g.*, 3 mg/mL; (9) any residual virus and DNA present in the

formulation of purified lysosomal sulfatase enzyme are removed by filtering through a viral filter and a DNA filter; and (10) a non-ionic surfactant, *e.g.*, polysorbate 20 (PS20 or Tween-20), is added to the formulation of purified lysosomal sulfatase enzyme. The final formulation of purified lysosomal sulfatase enzyme (the Bulk Drug Substance) is stored at 2-8°C or frozen. In ~~a particularly preferred embodiment~~one example, steps (8) to (10) are included in the purification process. In ~~a particularly preferred embodiment~~one example, the lysosomal sulfatase enzyme is recombinant human N-acetylgalactosamine-6-sulfatase (GALNS).

[0032] In some ~~embodiments~~examples, the harvest is collected in step (1) at a pH of about 6.5. In some ~~embodiments~~examples, the charcoal filter in step (1) is a Zeta Plus R55 Activated Carbon filter. In some ~~embodiments~~examples, the capture column in step (2) is a Zn-IMAC column. In some ~~embodiments~~examples, the Zn-IMAC column is a Zn-chelating Sepharose FF column. In some ~~embodiments~~examples, the filter in step (3) is a Mustang Q filter. In some ~~embodiments~~examples, the acid pH of the eluate or filtered eluate from step (2) or (3) is adjusted in step (4) to about 4.5 ± 0.1. In some ~~embodiments~~examples, the intermediate column in step (5) is a cation exchange column. In some ~~embodiments~~examples, the cation exchange column is a Fractogel EMD SE Hi-Cap column. In some ~~embodiments~~examples, the low pH of the eluate from the intermediate column from step (6) is adjusted in step (6) to about 3.5 ± 0.1. In some ~~embodiments~~examples, the polishing column in step (7) is a hydrophobic interaction chromatography (HIC) column. In some ~~embodiments~~examples, the HIC column is a ToyoPearl Butyl 650M column.

[0033] In some ~~embodiments~~examples, the formulation comprises 20 mM NaOAc/HOAc, 50 mM NaH_2PO_4, 30 mM arginine HCl, 2% (w/v) sorbitol, pH 5.4. In some ~~embodiments~~examples, the non-ionioc surfactant is polysorbate 20 (PS20). In some ~~embodiments~~examples, the concentration of lysosomal sulfatase enzyme in the formulation is adjusted to about 3 mg/mL. In some ~~embodiments~~examples, the viral filter is a DV20 filter and the DNA filter is a Mustang Q filter. In some ~~embodiments~~examples, the non-ionic surfactant added to the formulation is polysorbate 20 (PS20) to a final concentration of 0.01% (w/v).

[0033a] In yet another aspect, there is provided a formulation comprising

(a) recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) enzyme purified as defined above, said GALNS enzyme comprising an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, and

(i) having a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions,

(ii) having at least about 50% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and

(iii) having between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain,

wherein at least 98% of said GALNS enzyme is in the precursor form as determined by Coomassie Blue staining when subjected to SDS-PAGE under reducing conditions, and

(b) one or more pharmaceutically acceptable carriers, diluents or excipients comprising:

(i) NaOAc/HOAc and NaH_2PO_4 as buffering agents, wherein the concentration of NaOAc/HOAc is about 20 +/- 10 mM and the concentration of NaH_2PO_4 is about 50 +/-25 mM;

(ii) Arginine HCl, Tween-20 and sorbitol as stabilizers, wherein the concentration of Arginine HCl is about 30 +/- 20 mM, the concentration of Tween-20 is about 0.01% +/- 0.005% (w/v) and the concentration of sorbitol is about 2.0% +/- 1.0% (w/v); and

(iii) a pH of about 5.4 +/- 0.4.

[0034] Also disclosed is~~In a fifth aspect, the invention provides~~ a purified preparation of active highly phosphorylated recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) or biologically active mutant, variant or derivative thereof useful for treating a subject suffering from a lysosomal storage disease that is caused by (*e.g.*, Mucopolysaccharidosis type IVa (MPS IVa) or Morquio A syndrome) or associated with (*e.g.*, Multiple Sulfatase Deficiency (MSD)) a deficiency in the GALNS enzyme. In ~~a preferred embodiment~~one example, the purified preparation of active highly phosphorylated recombinant human GALNS has a GALNS

enzyme component having: (a) a purity of at least about 90%, 95%, 97%, 98%, or 99% as determined by Coomassie Blue staining or silver staining when subjected to SDS-PAGE under non-reducing conditions; (b) at least about 80%, 85%, 90%, or 95% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly) (position 79 of SEQ ID NO: 4); (c) N-linked glycosylation at the asparagine residues at positions 178 and 397, wherein some of the oligomannose chains attached to the asparagine residue at position 178 are bis-phosphorylated; (d) from 0.5 to 1.0, or from 0.5 to 0.9, or from 0.5 to 0.8, or from 0.5 to 0.75, or from 0.54 to 0.75 bis-phosphorylated oligomannose chains per monomeric protein chain (*e.g.*, at least 0.4, 0.45, 0.5, 0.55, 0.6, or 0.65 and up to 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.98, or 1.0 bis-phosphorylated oligomannose chains per monomeric protein chain, or any combination of any of these numbers); and (e) at least 65%, or at least 70% (*e.g.*, at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, 98.5%, 99% or 99.5%) of the GALNS enzyme is in the precursor form as determined by Coomassie Blue staining when subjected to SDS-PAGE under reducing conditions or by SDS-capillary gel electrophoresis (SDS-CGE). In addition, the GALNS enzyme may optionally also (f) exhibit a specific activity that is at least about 30% (*e.g.*, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 2-fold, 2.5-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold or 50-fold) greater than the specific activity of a control GALNS enzyme of the same amino acid sequence that has been produced in host cells (*e.g.*, CHO cells or CHO-derived cells) that do not express recombinant human SUMF1. Optionally, the GALNS enzyme exhibits a specific uptake (Kuptake) into fibroblasts that is about 0.1 to 10 nM, or about 0.1 to 7 nM, or about 0.5 to 5 nM, or about 1 to 5 nM, or about 1 to 3.5 nM, about 1 nM, about 1.5 nM, about 2 nM, about 2.5 nM, about 3 nM or about 3.5 nM, or any combination of any of these numbers.

[0035] The purified, active highly phosphorylated recombinant human GALNS consists of a major band of about 55-60 kDa (*i.e.*, precursor human GALNS being at least about 75%, or at least 80%, preferably at least about 85%, more preferably at least about 90%, and even more preferably at least about 95%, at least 97%, at least 98%, at least 98.5%, at least 99%, or at least 99.5% of the visible proteins) and minor bands at ~39 kDa and ~19 kDa (*i.e.*, mature or processed human GALNS being less than about 25%, less than about 20%, preferably less than about 15%, more preferably less than about 10%, and even more preferably less than about 5%, less than about 3%, less than about 2%, less than about 1.5%, less than about 1% or less than about 0.5% of the visible proteins) when subjected to SDS-PAGE under reducing conditions, or as determined by SDS-CGE. In once ~~a particularly preferred~~

~~embodiment~~example, the purified, active highly phosphorylated recombinant human GALNS consists essentially of a single band of about 55-60 kDa (*i.e.*, precursor human GALNS) when subjected to SDS-PAGE under reducing conditions, or as determined by SDS-CGE. In one ~~embodiment~~example, the purified, active highly phosphorylated recombinant human GALNS is useful for treating MPS IVa or Morquio A syndrome. In one ~~embodiment~~example, the purified, active highly phosphorylated recombinant human GALNS is useful for treating MSD.

[0036] Also disclosed is~~In a sixth aspect, the invention provides~~ a method of treating diseases caused all or in part by deficiency, or are associated with a deficiency, of a lysosomal sulfatase enzyme. The method comprises administering a therapeutic recombinant human lysosomal sulfatase enzyme produced by the methods of the present ~~invention~~disclosure, wherein the lysosomal sulfatase enzyme binds to an MPR receptor and is transported across the cell membrane, enters the cell and is delivered to the lysosomes within the cell.

[0037] In one ~~embodiment~~example, the method comprises treating a subject suffering from a deficiency of a lysosomal sulfatase enzyme comprising administering to the subject in need thereof a therapeutically effective amount of said lysosomal sulfatase enzyme, wherein said lysosomal sulfatase enzyme is a recombinant human lysosomal sulfatase enzyme or biologically active fragment, mutant, variant or derivative thereof produced by a CHO-derived END3 complementation group cell or a derivative thereof. In some ~~embodiments~~examples, the method comprises administering a therapeutic recombinant human lysosomal sulfatase enzyme, or a biologically active fragment, mutant, variant or derivative thereof, alone or in combination with a pharmaceutically acceptable carrier, diluent or excipient. ~~Preferred embodiments~~Some examples include optimizing the dosage to the needs of the subjects to be treated, preferably mammals and most preferably humans, to most effectively ameliorate the deficiency of the lysosomal sulfatase enzyme.

[0038] Such therapeutic lysosomal sulfatase enzymes are particularly useful, for example, in the treatment of patients suffering from lysosomal storage diseases caused by a deficiency of a lysosomal sulfatase enzyme, such as patients suffering from Metachromatic Leukodystrophy or MLD, Mucopolysaccharidosis type VI (MPS VI) or Maroteaux-Lamy syndrome, Mucopolysaccharidosis type II (MPS II) or Hunter syndrome, Mucopolysaccharidosis type IIIa (MPS IIIa) or Sanfilippo A syndrome, Mucopolysaccharidosis type IIId (MPS IIId) or Sanfilippo D syndrome, and Mucopolysaccharidosis type IVa (MPS IVa) or Morquio A syndrome. In ~~a particularly preferred embodiment~~one example, the lysosomal storage disease is MPS IVa or Morquio A syndrome

and the lysosomal sulfatase enzyme is recombinant human N-acetylgalactosamine-6-sulfatase (GALNS). In yet other ~~embodiments~~examples, the ~~invention~~present disclosure also provides pharmaceutical compositions comprising the deficient lysosomal sulfatase enzyme causing the lysosomal storage disease and a pharmaceutically acceptable carrier, diluent or excipient.

[0039] In another ~~embodiment~~example, the method comprises treating a subject suffering from a lysosomal storage disease that is associated with a deficiency in one or more lysosomal sulfatase enzymes comprising administering to the subject in need thereof a therapeutically effective amount of a lysosomal sulfatase enzyme, wherein said lysosomal sulfatase enzyme is a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) or biologically active fragment, mutant, variant or derivative thereof produced by a CHO-derived END3 complementation group cell or a derivative thereof. In some ~~embodiments~~examples, the method comprises administering therapeutic recombinant human GALNS enzyme or a biologically active fragment, mutant, variant or derivative thereof alone or in combination with a pharmaceutically acceptable carrier, diluent or excipient. In ~~a particularly preferred embodiment~~one example, the lysosomal storage disease is Multiple Sulfatase Deficiency (MSD).

[0040] In ~~particularly preferred embodiments~~another example, the CHO-derived END3 complementation group cell or a derivative thereof is a G71 cell line, a G71S cell line or a G71 or G71S derivative thereof.

[0041] In still another ~~embodiment~~example, the present ~~invention~~disclosure provides for a method of enzyme replacement therapy by administering a therapeutically effective amount of lysosomal sulfatase enzyme to a subject in need of the enzyme replacement therapy, wherein the cells of the patient have lysosomes which contain insufficient amounts of the lysosmal sulfatase enzyme to prevent or reduce damage to the cells, whereby sufficient amounts of the lysosomal sulfatase enzyme enter the lysosomes to prevent or reduce damage to the cells. The cells may be within or without the CNS or need not be set off from the blood by capillary walls whose endothelial cells are closely sealed to diffusion of an active agent by tight junctions.

[0042] In a particular ~~embodiment~~example, the ~~invention~~present disclosure provides compositions and pharmaceutical compositions comprising an active recombinant human lysosomal sulfatase enzyme having a biological activity which is reduced, deficient, or absent in the target lysosome and which is administered to the subject. Preferred active human lysosomal sulfatase enzymes include, but are not limited to, arylsulfatase A, arylsulfatse B, iduronate-2-

sulfatase, sulfamidase/heparan-N-sulfatase, N-acetylglucosamine-6-sulfatase, and N-acetylgalactosamine-6-sulfatase. In ~~a preferred embodiment~~one example, N-acetylgalactosamine-6-sulfatase is the active recombinant human lysosomal sulfatase enzyme.

[0043] In ~~a preferred embodiment~~one example, the ~~invention~~present disclosure provides a method of treating a subject suffering from MPS IVa or Morquio A syndrome, or MSD, by administering to the subject a therapeutically effective amount of any of the recombinant human N-acetylgalactosamine-6-sulfatase (GALNS), purified preparations and/or sterile compositions described herein.

[0044] In ~~a more preferred embodiment~~one example, the ~~invention~~present disclosure provides a method of treating a subject suffering from MPS IVa or Morquio A syndrome, or MSD, by administering to the subject a therapeutically effective amount of recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) produced by END3 complementation group cells, wherein the recombinant human GALNS has a high level of conversion of the active site cysteine residue to C_α-formylglycine (FGly) (*i.e.*, at least about 50%, preferably at least about 70%, more preferably at least about 90%, even more preferably at least about 95% conversion), and high levels of phosphorylation (*i.e.*, greater than about 0.25, preferably greater than 0.5, and more preferably greater than about 0.75 bis-phosphorylated oligomannose chains per protein chain).

[0045] In ~~a particularly preferred embodiment~~one example, the ~~invention~~present disclosure provides a method of treating a subject suffering from MPS IVa or Morquio A syndrome, or MSD, by administering to the subject a therapeutically effective amount of a purified, active highly phosphorylated recombinant human GALNS preparation that has a GALNS enzyme component having: (a) a purity of at least about 90%, 95%, 97%, 98% or 99% as determined by Coomassie Blue staining or silver staining when subjected to SDS-PAGE under non-reducing conditions; (b) at least about 80%, 85%, 90% or 95% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly) (position 79 of SEQ ID NO: 4); (c) from 0.5 to 1.0, or from 0.5 to 0.9, or from 0.5 to 0.8, or from 0.5 to 0.75, or from 0.54 to 0.75 bis-phosphorylated oligomannose chains per monomeric protein chain (*e.g.*, at least 0.4, 0.45, 0.5, 0.55, 0.6 or 0.65 and up to 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.98, or 1.0 bis-phosphorylated oligomannose chains per monomeric protein chain, or any combination of any of these numbers); and (d) at least 65%, or at least 70% (*e.g.*, at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, 98.5%, 99% or 99.5%) of the GALNS enzyme is in the precursor form as determined by Coomassie Blue staining when subjected to SDS-PAGE under reducing conditions or by SDS-capillary gel electrophoresis (SDS-CGE). In addition, the GALNS enzyme may optionally also (e) exhibit

a specific activity that is at least about 30% (e.g., 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 2-fold, 2.5-fold, 3-fold, 4-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold or 50-fold) greater than the specific activity of a control GALNS enzyme of the same amino acid sequence that has been produced in host cells (*e.g.*, CHO cells or CHO-derived cells) that do not express recombinant human SUMF1. Optionally, the GALNS enzyme exhibits a specific uptake (Kuptake) into fibroblasts that is about 0.1 to 10 nM, or about 0.1 to 7 nM, or about 0.5 to 5 nM, or about 1 to 5 nM, or about 1 to 3.5 nM, about 1 nM, about 1.5 nM, about 2 nM, about 2.5 nM, about 3 nM or about 3.5 nM, or any combination of any of these numbers.

[0046] The purified, active highly phosphorylated recombinant human GALNS consists of a major band of about 55-60 kDa (*i.e.*, precursor human GALNS being at least about 75%, or at least about 80%, preferably at least about 85%, more preferably at least about 90%, and even more preferably at least about 95%, at least about 97%, at least about 98%, at least about 98.5%, at least about 99% or at least about 99.5% of the visible proteins) and minor bands at ~39 kDa and ~19 kDa (*i.e.*, mature or processed human GALNS being less than about 25%, or less than about 20%, preferably less than about 15%, more preferably less than about 10%, and even more preferably less than about 5%, less than about 3%, less than about 2%,less than about 1.5%, less than about 1% or less than about 0.5% of the visible proteins) when subjected to SDS-PAGE under reducing conditions, or as determined by SDS-CGE. In ~~a more particularly preferred embodiment~~one example, the purified, active highly phosphorylated recombinant human GALNS consists essentially of a single band of about 55-60 kDa (*i.e.*, precursor human GALNS) when subjected to SDS-PAGE under reducing conditions, or as determined by SDS-CGE.

[0047] In some ~~embodiments~~examples, the subject is suffering from MPS IVa or Morquio A syndrome. In some ~~embodiments~~examples, the subject is suffering from MSD.

[0048] Corresponding use of active highly phosphorylated lysosomal sulfatase enzymes of the ~~invention~~present disclosure, which are preferably produced by methods of the ~~invention~~present disclosure, in preparation of a medicament for the treatment of the lysosomal storage diseases described above is also contemplated.

[0049] Also disclosed are~~In a seventh aspect, the present invention provides~~ pharmaceutical compositions comprising an active highly phosphorylated recombinant human lysosomal sulfatase enzyme as described hereinabove which is useful for treating diseases caused all or in part by, or are

associated with, the deficiency in such lysosomal sulfatase enzyme, and one or more pharmaceutically acceptable carriers, diluents or excipients. In ~~a preferred embodiment~~one example, the pharmaceutical composition comprises an active highly phosphorylated recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) or biologically active fragment, mutant, variant or derivative thereof produced by the methods of the ~~invention~~present disclosure and one or more pharmaceutically acceptable carriers, diluents or excipients. Such pharmaceutical compositions may be suitable for administration by several routes such as intrathecal, parenteral, topical, intranasal, inhalational or oral administration. In ~~a preferred embodiment~~one example, the pharmaceutical compositions are suitable for parenteral administration. Within the scope of this ~~aspect are embodiments~~examples featuring nucleic acid sequences encoding the full-length lysosomal sulfatase enzymes or fragments, mutants, variants or derivatives thereof, which may be administered *in vivo* into cells affected with a lysosomal enzyme deficiency.

[0050] In ~~a more preferred embodiment~~one example, the pharmaceutical composition comprises an active highly phosphorylated recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) or biologically active fragment, mutant, variant or derivative thereof produced by the methods of the ~~invention~~present disclosure and one or more pharmaceutically acceptable carriers, diluents or excipients in a formulation comprising one or more buffering agents, and one or more stabilizers. In certain ~~embodiments~~examples, the composition comprises an amount of phosphate buffer effective to reduce dephosphorylation of said GALNS enzyme; and a stabilizing amount of one or more stabilizers selected from the group consisting of amino acid salts, amino acid buffers, surfactants and polyols; wherein said formulation is at a pH of about 5.0-5.8.

[0051] In some ~~embodiments~~examples, the GALNS enzyme comprises an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, and has: (i) a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions, (ii) at least about 80% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and (iii) optionally, between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain, wherein at least 70% of said GALNS enzyme is in the precursor form as determined by Coomassie Blue staining when subjected to SDS-PAGE under reducing conditions. In some ~~embodiments~~examples, the GALNS enzyme is at least 95% pure as determined by RP-HPLC. In some ~~embodiments~~examples, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 98.5%, at least 99% or at least 99.5% of the GALNS enzyme is in the precursor form as determined by Coomassie

Blue staining when subjected to SDS-PAGE under reducing conditions. In some ~~embodiments~~examples, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 98.5%, at least 99% or at least 99.5% of the GALNS enzyme is in the precursor form as determined by SDS-capillary gel electrophoresis. In some ~~embodiments~~examples, the GALNS enzyme has at least about 90% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly). In some ~~embodiments~~examples, between 50% to 80% of the GALNS enzyme binds to a mannose-6-phosphate receptor column. In some ~~embodiments~~examples, the GALNS enzyme exhibits a specific uptake (Kuptake) into fibroblasts that is about 1 to 5 nM. In some ~~embodiments~~examples, the GALNS enzyme exhibits a specific uptake (Kuptake) into fibroblasts that is about 1 to 3.5 nM.

[0052] The concentration of GALNS or biologically active fragment, mutant, variant or derivative thereof in the formulation is from about 0.1 to 10 mg/mL, preferably from about 0.5 to 5 mg/mL and more preferably from about 0.5 to 1.5 mg/mL.

[0053] In certain ~~embodiments~~examples, formulation comprises an amount of phosphate buffer effective to reduce dephosphorylation of said GALNS enzyme. In related ~~embodiments~~examples, the phosphate buffer is NaH_2PO_4 or its equivalent. In a further ~~embodiment~~example, the formulation further comprises a second buffer. In one ~~embodiment~~example the second buffer is an acetate buffer. In another ~~embodiment~~example, the acetate buffer is NaOAc/HOAc or its equivalent. Exemplary buffers are described in greater detail in the Detailed Description.

[0054] It is contemplated that the concentration of NaOAc/HOAc or its equivalent in the formulation is from about 5 to 100 mM, preferably from about 5 to 50 mM, and more preferably from about 10 to 30 mM. In a related ~~embodiment~~example, the concentration of NaH_2PO_4 or its equivalent in the formulation is from about 5 to 100 mM, preferably from about 25 to 100 mM, and more preferably from about 25 to 75 mM. In certain ~~embodiments~~examples, the pH of the formulation is about pH 4.5-6.5, preferably about pH 5.0-6.0, and more preferably about pH 5.0-5.8.

[0055] In still another ~~embodiment~~example, the formulation comprises a stabilizing amount of one or more stabilizers selected from the group consisting of amino acid salts, amino acid buffers, surfactants and polyols. In one ~~embodiment~~example, the stabilizer is an arginine or histidine salt or buffer, optionally arginine hydrochloride. In a related ~~embodiment~~example, the stabilizer is a polysorbate, optionally polysorbate 20. In a further ~~embodiment~~example, the stabilizer is a trihydric

or higher sugar alcohol, optionally sorbitol. Exemplary stabilizers are described in greater detail in the Detailed Description.

[0056] In certain ~~embodiments~~examples, the stabilizers are selected from Arginine HCl or its equivalent, Tween-20 (Polysorbate 20) or its equivalent, and sorbitol or its equivalent. In some ~~embodiments~~examples, the concentration of Arginine HCl or its equivalent in the formulation is from about 5 to 200 mM, preferably from about 10 to 100 mM, and more preferably from about 10 to 50 mM. In another ~~embodiment~~example, the concentration of Tween-20 or its equivalent in the formulation is from about 0.001 to 1.0% (w/v), preferably from about 0.005 to 0.2 % (w/v), and more preferably from about 0.005 to 0.015% (w/v). In a related ~~embodiment~~example, the concentration of sorbitol or its equivalent in the formulation is from about 0.1 to 10% (w/v), preferably from about 0.5 to 5 % (w/v), and more preferably from about 1.0 to 3.0% (w/v). In one ~~embodiment~~example, the formulation comprises an arginine salt or buffer, a polysorbate, and a polyol.

[0057] The ~~invention~~present disclosure further provides a method of preventing dephosphorylation of a recombinant human GALNS enzyme comprising mixing GALNS enzyme and a phosphate buffer, to a final concentration of phosphate buffer that is between about 25 mM and 75 mM. In ~~exemplary embodiments~~one example, the amount of dephosphorylation is reduced compared to a formulation of the same enzyme in 1 mM phosphate buffer, e.g. when tested after 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months or 6 months of storage at room temperature (e.g. 25 °C).

[0057a] In another aspect, there is provided the use of a composition comprising a purified recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) enzyme purified as defined above in the manufacture of a medicament for treating a subject suffering from Mucopolysaccharidosis type IVa (MPS IVa) or Morquio A syndrome, said GALNS enzyme comprising an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, wherein said GALNS enzyme:

(i) has a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions,

(ii) has at least about 50% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and

(iii) optionally, has between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain,

and wherein at least 98% of said GALNS enzyme is in the precursor form as determined by SDS-CGE.

[0058] In ~~a particularly preferred embodiment~~one example, the pharmaceutical composition comprises an active highly phosphorylated recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) or biologically active fragment, mutant, variant or derivative thereof produced by the methods of the ~~invention~~present disclosure and one or more pharmaceutically acceptable carriers, diluents or excipients in a formulation comprising NaOAc/HOAc and NaH_2PO_4 as buffering agents, and Arginine HCl, Tween-20 (Polysorbate 20) and Sorbitol as stabilizers. The concentration of GALNS in the formulation is about 1.0 +/- 0.5 mg/mL. The concentration of NaOAc/HOAc in the formulation is about 20 +/- 10 mM, and the concentration of NaH_2PO_4 in the formulation is about 50 +/- 25 mM. The pH of the formulation is pH 5.4 +/- 0.4. The concentration of Arginine HCl in the formulation is about 30 +/- 20 mM. The concentration of Tween-20 in the formulation is about 0.01 +/- 0.005% (w/v). The concentration of sorbitol in the formulation is about 2.0 +/- 1.0% (w/v).

[0059] Also disclosed is~~In another aspect, the invention provides~~ a method for detecting activity of a lysosomal sulfatase enzyme comprising (a) culturing chondrocyte cells from a patient suffering from lysosomal sulfatase enzyme deficiency, e.g., a patient suffering from Morquio syndrome, under conditions that promote maintenance of chondrocyte differentiation; (b) contacting the chondrocytes with a lysosomal sulfatase enzyme that degrades keratan sulfate; and (c) detecting levels of keratan sulfate in the cells, wherein a reduced keratan sulfate level in cells contacted with the lysosomal sulfatase enzyme compared to cells not contacted with the lysosomal sulfatase enzyme is indicative of lysosomal sulfatase enzyme activity. In some ~~embodiments~~examples, the lysosomal sulfatase enzyme is N-acetylgalactosamine-6-sulfatase (GALNS). In some ~~embodiments~~examples, the culturing is carried out in media comprising insulin growth factor 1 (IGF1), transforming growth factor beta (TGF-β), transferrin, insulin and ascorbic acid. In some ~~embodiments~~examples, the keratan sulfate is detected by confocal microscopy, or via binding to anti-keratan sulfate antibody. The method may be carried out with any lysosomal sulfatase enzyme, including naturally occurring or recombinant human enzyme, or fragments or variants thereof, including variants comprising an amino acid sequence at least 80%, 85%, 90%, 95% or 100% identical to the precursor human enzyme, without signal sequence, or the mature form thereof.

[0060] Also disclosed is~~In yet another aspect, the invention provides~~ a cell-based assay for measuring the activity of a recombinant human lysosomal enzyme to degrade natural substrates. The method comprises (a) culturing an isolated human cell deficient in the lysosomal enzyme under conditions in which natural substrates for the lysosomal enzyme accumulate; (b) contacting the cell with the lysosomal enzyme; (c) lysing the cell; (d) adding to the cell lysate an enzyme that (i) is specific for the natural substrates, and (ii) cleaves small oligosaccharides from the natural substrates; (e) labeling the small oligosaccharides with a detectable moiety; (f) optionally separating the labeled small oligosaccharides; (g) detecting the labeled small oligosaccharides; and (h) determining the activity of the lysosomal enzyme to degrade the natural substrates by comparing (i) the amount of labeled small oligosaccharide from cells contacted with the lysosomal enzyme with (ii) the amount of labeled small oligosaccharides from cells not contacted with the lysosomal enzyme, wherein a reduction in (h)(i) as compared to (h)(ii) indicates the activity of the lysosomal enzyme to degrade natural substrates. In one ~~embodiment~~example, the small oligosaccharide is a mono-, di, or tri-saccharide. In a related ~~embodiment~~example, the small oligosaccharide is a disaccharide. In some ~~embodiments~~examples, the lysosomal enzyme is selected from the group consisting of arylsulfatase B (ARSB),

iduronate-2-sulfatase (IDS), sulfamidase/heparin-N-sulfatase (SGSH), N-acetylglucosamine-sulfatase (G6S) and N-acetylgalactosamine-6-sulfatase (GALNS). In some ~~embodiments~~examples, the lysosomal enzyme is α-L-iduronidase (IDU). In some ~~embodiments~~examples, the lysosomal enzyme is acid α-glucosidase (GAA). In some ~~embodiments~~examples, the lysosomal enzyme is β-glucoronidase (GUSB). In some ~~embodiments~~examples, the lysosomal enzyme is β-galactosidase (GLB1).

[0061] Suitable human cells that can be used in the cell-based assay include any human cell that is deficient in the lysosomal enzyme to be tested, such that can accumulate the natural substrates for the lysosomal enzyme. For example, cells naturally exhibiting a full (100%) or partial deficiency in activity, e.g., 30%, 50%, 70%, 80%, 90%, 95% reduction or more in activity, may be used. Cells expressing a mutant enzyme with diminished activity, or cells derived from patients suffering from a lysosomal storage disease, e.g. a mucopolysaccharidosis, may be used. Cells recombinantly altered to knockout or reduce lysosomal enzyme activity, e.g. through introducing a mutation to the encoding gene or its promoter or other regulatory region, may be used. Cells treated to reduce lysosomal enzyme activity, e.g. treated with antisense or RNAi to reduce enzyme expression, may be used.

[0062] Suitable enzymes that cleave (digest) small oligosaccharides from carbohydrates and that are "specific for" (i.e. predominantly digest) the natural substrates of the lysosomal enzyme may be selected by those of ordinary skill in the art. For example, for detection of activity of GALNS or GLB1 (enzymes that degrades keratan sulfate) the enzyme of step (d) may be Keratanase II or any enzyme that acts primarily on keratan sulfate. As another example, for detection of IDU, ARSB, IDS or GUSB (enzymes that degrade dermatan sulfate), the enzyme of step (d) may be Chondroitinase ABC or any enzyme that acts primarily on dermatan sulfate. As another example, for detection of IDU, IDS, SGHS, G6S or GUSB (enzymes that degrade heparan sulfate), the enzyme of step (d) may be Heparanase I or Heparanase II, or both. As yet another example, for detection of GAA (an enzyme that degrades glycogen), the enzyme of step (d) may be α-amylase or any enzyme that acts primarily on glycogen.

[0063] This cell-based method is capable of great sensitivity in detecting lysosomal enzyme activity. In some ~~embodiments~~examples, the lysosomal enzyme activity is detectable when the concentration of lysosomal enzyme is as low as about 10 nM, or about 5 nM, or about 1 nM, or about 0.75 nM, or about 0.5 nM, or about 0.25 nM, or about 0.1 nM, or about 0.05 nM, or about 0.01 nM, or about 0.005 nM, or about 1 pM, or about 0.5 pM.

WHAT IS CLAIMED:

1. A method of purifying a recombinant human N-acetylgalactosamine-6-sulfatase (GALNS) enzyme, said GALNS enzyme comprising an amino acid sequence at least 95% identical to amino acids 27 to 522 of SEQ ID NO:4, wherein said GALNS enzyme:

(i) has a purity of at least about 95% as determined by Coomassie Blue staining when subjected to SDS-PAGE under non-reducing conditions,

(ii) has at least about 50% conversion of the cysteine residue at position 53 to C_α-formylglycine (FGly), and

(iii) has between 0.5 to 0.8 bis-phosphorylated oligomannose chains per monomeric protein chain,

and wherein at least 98% of said GALNS enzyme is in the precursor form as determined by SDS-CGE, comprising:

a) filtering a culture medium containing the GALNS enzyme secreted from a mammalian cell line that expresses human sulfatase modifying factor 1 (SUMF1) and the recombinant human GALNS enzyme,

ultrafiltering/diafiltering the filtered culture medium, whereby the ultrafiltered/diafiltered culture medium is concentrated about 20X, and charcoal filtering the 20 X concentrated ultrafiltered/diafiltered culture medium;

b) loading the charcoal filtered 20X ultrafiltered/dialfiltered culture medium from step a) onto a Zn-chelating Sepharose FF capture column, washing the column under conditions such that the GALNS enzyme is retained on the column, and eluting the GALNS enzyme from the column;

c) optionally, filtering the eluate from the Zn-chelating Sepharose FF capture column in step b) through a filter to remove viruses;

d) adjusting the pH of the eluate from the Zn-chelating Sepharose FF capture column from step b) or the filtrate from step c) to about pH 4.5 ± 0.1, and filtering the pH 4.5-adjusted eluate from the Zn-chelating Sepharose FF capture column or filtrate;

e) loading the pH 4.5-adjusted eluate from the Zn-chelating FF capture column or filtrate from step d) onto a Fractogel EMD SE Hi-Cap cation exchange column, washing the column under conditions such that the GALNS enzyme is retained on the column, and eluting the GALNS enzyme from the column;