

Description

Title of Invention: NOVEL LYOPHILIZED FORMULATION CONTAINING α -GALACTOSIDASE A FUSION PROTEIN

Technical Field

- [1] The present invention relates to a lyophilized formulation containing α -galactosidase A fusion protein, a preparation method therefor, and use thereof.

[2]

Background Art

- [3] Lysosomes are intracellular organelles involved in the degradation of proteins, various lipids such as glycolipids and cholesterol, and carbohydrates and the recycling of degraded products as primary constituents of new proteins, membrane components, and other molecules. Examples of diseases associated with the functions of lysosomes are lysosomal storage diseases (LSDs).

- [4] Lysosomal storage diseases (LSDs) are caused by defects in genes encoding enzymes of degrading glycolipid or polysaccharide waste products in cellular lysosomes, and the biological activity of lysosomal enzymes is significantly reduced or almost absent in cells and tissues of individuals with lysosomal storage diseases. Such a deficiency of degrading enzymes causes the accumulation of substances in large quantities without degradation, ultimately causing problems in cellular functions.

- [5] Fabry disease, known as one of the lysosomal storage diseases, is a type of congenital metabolic disorder of glycolipids (glycosphingolipids) resulting from deficient or insufficient activity of alpha (α)-galactosidase A, which is a hydrolase present in lysosomes. Fabry disease, as a representative disease of α -galactosidase A deficiency, is an X chromosome-associated disease. Typical Fabry disease subjects generally have α -galactosidase A activity of less than 1% and show a wide range of symptoms including severe pain in the extremities (acroparesthesia), hypersensitivity to cold, corneal and lenticular changes, skin lesions (angiokeratoma), renal failure, cardiovascular disease, pulmonary failure, nervous system symptoms, and stroke.

- [6] Fabry disease causes a progressive accumulation of globotriaosylceramide (Gb3) in most of the tissues of the body. The accumulation of Gb3 is mainly found in vascular endothelia. Such progressive accumulation of Gb3 in vascular endothelia leads to ischemia and infarction in organs, such as the kidneys, heart, or brain.

[7]

- [8] Enzyme-replacement therapy (ERT) is a representative method for treating lysosomal storage diseases, and a number of related studies have been conducted (Frances M. Platt et al., J Cell Biol. 2012 Nov 26;199(5):723-34). Specifically, lysosomal storage

diseases are caused by genetic defects in particular enzymes, and thus an enzyme-replacement therapy for deficient enzymes is essential. Enzyme-replacement therapy is a standard therapy in lysosomal storage diseases and may exhibit an effect of alleviating existing symptoms or delaying the progress of the diseases by replacing the deficient enzymes. Therefore, studies have been conducted on various formulations including α -galactosidase A for preventing and treating α -galactosidase A deficiency.

- [9] However, the composition of a formulation for exhibiting stability significantly varies depending on the structure, physicochemical properties, dosage, etc., of an active ingredient (e.g., protein drug), and thus the development of a particular composition suitable for the active ingredient is essentially required, and the development of a formulation containing α -galactosidase A is insufficient. Particularly, a lyophilized formulation containing an α -galactosidase A fusion protein at a high concentration exhibits significantly low storage stability due to issues such as protein precipitation, and thus conventional lyophilized formulations contain an α -galactosidase A fusion protein at a concentration of 5 mg/mL or less. However, there is a need to develop a highly stable formulation containing an α -galactosidase A fusion protein at a high concentration to increase efficacy.

[10]

Disclosure of Invention

Technical Problem

- [11] Moreover, developing lyophilized formulations that are suitable for long-term storage while maintaining the structure and activity of proteins is challenging due to issues such as the loss of structure or function of proteins during the lyophilization of protein medicines. Therefore, there is still a need to develop a lyophilized formulation capable of increasing the protein recovery rate while ensuring stability even for long-term storage.

[12]

Solution to Problem

- [13] An aspect of the present invention is to provide a lyophilized formulation containing an α -galactosidase A fusion protein.
- [14] Another aspect of the present invention is to provide a method for preparing the lyophilized formulation.
- [15] Still another aspect of the present invention is to provide a method for reconstituting the lyophilized formulation.

Advantageous Effects of Invention

- [16] The lyophilized formulation according to the present invention can stably store an α -galactosidase A fusion protein for a long time and can administer to a patient

an appropriate dosage of the fusion protein by increasing the recovery rate in the reconstitution.

[17]

Brief Description of Drawings

[18] FIG. 1 compares the cake appearances of a lyophilized formulation immediately after dissolution and three days after dissolution according to pH conditions.

[19] FIG. 2 compares the recovery rates of the native form of the lyophilized formulation according to pH conditions.

[20] FIGS. 3 and 4 compare the appearances of lyophilized formulations containing a sugar or sugar alcohol.

[21] FIG. 5 compares the recovery rates of the native form of the lyophilized formulation at pH 6.5 according to the addition of sugar or sugar alcohol.

[22] FIG. 6 compares the recovery rate of the native form of the lyophilized formulation at pH 6.8 according to the addition of sugar or sugar alcohol.

[23] FIG. 7 compares the appearances of a lyophilized formulation according to concentration changes of a sugar (sucrose).

[24] FIG. 8 compares the recovery rates of the native form of a lyophilized formulation according to concentration changes of a sugar (sucrose).

[25] FIG. 9 examines the appearance of a lyophilized formulation with an optimum composition under refrigeration for 12 months.

[26] FIG. 10 compares the recovery rate of the native form of a lyophilized formulation at 3, 6, and 12 months.

[27]

Best Mode for Carrying out the Invention

[28] In accordance with one aspect of the present invention, there is provided a lyophilized formulation containing an α -galactosidase A fusion protein.

[29] In one embodiment, the lyophilized formulation may include a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region; a buffer; and a sugar or sugar alcohol.

[30] In another embodiment, the lyophilized formulation may include a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein at a concentration of 1 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; and a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL.

[31] In the lyophilized formulation of any one of the previous embodiments, the lyophilized formulation may include a mixture obtained by lyophilizing an aqueous solution containing: the fusion protein at a concentration of 1 to 40 mg/mL; a buffer

with a pH of 6.5 to 8.0; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; and an amino acid at a concentration of 1.0 to 3.0% (w/v).

- [32] In the lyophilized formulation of any one of the previous embodiments, the lyophilized formulation may include a mixture obtained by lyophilizing an aqueous solution containing: the fusion protein at a concentration of 1 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; and a non-ionic surfactant at a concentration of 0.001 to 0.1% (w/v).
- [33] In the lyophilized formulation of any one of the previous embodiments, the lyophilized formulation may include a mixture obtained by lyophilizing an aqueous solution containing: the fusion protein at a concentration of 1 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0 containing 5 to 30 mM histidine; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; polysorbate 20 at a concentration of 0.001 to 0.1% (w/v); and serine at a concentration of 1.0 to 3.0% (w/v).
- [34] In the lyophilized formulation of any one of the previous embodiments, the sugar may be glucose, fructose, galactose, lactose, maltose, sucrose, trehalose, or a combination thereof.
- [35] In the lyophilized formulation of any one of the previous embodiments, the sugar alcohol may be mannitol, sorbitol, or a combination thereof.
- [36] In the lyophilized formulation of any one of the previous embodiments, the aqueous solution may further contain an amino acid at a concentration of 1.0 to 3.0% (w/v).
- [37] In the lyophilized formulation of any one of the previous embodiments, the amino acid may be selected from the group consisting of serine, arginine, threonine, glutamine, glycine, alanine, and a combination thereof.
- [38] In the lyophilized formulation of any one of the previous embodiments, the aqueous solution may further contain a non-ionic surfactant at a concentration of 0.001 to 0.1% (w/v).
- [39] In the lyophilized formulation of any one of the previous embodiments, the non-ionic surfactant may be selected from the group consisting of poloxamer 188, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, and a combination thereof.
- [40] In the lyophilized formulation of any one of the previous embodiments, the buffer may contain histidine or a salt thereof, citric acid or a salt thereof, acetic acid or a salt thereof, phosphoric acid or a salt thereof, or a combination thereof.
- [41] In the lyophilized formulation of any one of the previous embodiments, the buffer may contain 5 to 30 mM histidine.
- [42] In the lyophilized formulation of any one of the previous embodiments, the lyophilized formulation may not further contain an isotonic agent.
- [43] In the lyophilized formulation of any one of the previous embodiments, the isotonic agent may be sodium chloride.

- [44] In the lyophilized formulation of any one of the previous embodiments, the α -galactosidase A may include the amino acid sequence of SEQ ID NO: 1.
- [45] In the lyophilized formulation of any one of the previous embodiments, the immunoglobulin Fc region may include the amino acid sequence of SEQ ID NO: 3.
- [46] In the lyophilized formulation of any one of the previous embodiments, the α -galactosidase A fusion protein may include the amino acid sequence of SEQ ID NO: 4.
- [47] In the lyophilized formulation of any one of the previous embodiments, the immunoglobulin Fc region may be derived from IgG4.
- [48] In the lyophilized formulation of any one of the previous embodiments, the immunoglobulin Fc region may be aglycosylated.
- [49] In the lyophilized formulation of any one of the previous embodiments, the fusion protein may have a structure in which two molecules of α -galactosidase A are linked to monomers of an immunoglobulin Fc region in a dimeric form, respectively.
- [50] In the lyophilized formulation of any one of the previous embodiments, the lyophilized formulation may be used to prevent or treat Fabry disease.
- [51] In accordance with another aspect of the present invention, there is provided a method for preparing the lyophilized formulation.
- [52] In an embodiment, the preparation method may include lyophilizing an aqueous solution containing: i) an α -galactosidase A fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region; ii) a buffer; and iii) a sugar or sugar alcohol.
- [53] In another embodiment, the preparation method of any one of the previous embodiments may include further mixing an amino acid, a non-ionic surfactant, a preservative, or a combination thereof with the aqueous solution.
- [54] In the preparation method of any one of the previous embodiments, the method may include lyophilizing 1 to 10 mL of an aqueous solution containing at least one of a protein, a buffer, a sugar, a sugar alcohol, an amino acid, a non-ionic surfactant, a preservative, or a combination thereof.
- [55] In accordance with another aspect of the present invention, there is provided a method for reconstituting the lyophilized formulation, the method including adding a reconstitution solution to the lyophilized formulation.
- [56] In an embodiment, the reconstitution solution may be distilled water.
- [57] In another embodiment, the reconstituted formulation may contain an α -galactosidase A fusion protein at a concentration of 10 to 100 mg/mL.
- [58]

Mode for the Invention

- [59] Hereinafter, detailed contents for implementing the present invention will be described below.

- [60] Each description and embodiment disclosed in the present application may also be applied to other descriptions and embodiments. That is, all combinations of various elements disclosed in the present application fall within the scope of the present disclosure. Furthermore, the scope of the present invention is not limited by the specific description below.
- [61] Furthermore, a skilled in the art will recognize, or be able to ascertain, by using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Furthermore, these equivalents are intended to be included in the present invention.
- [62] Throughout the description herein, not only the typical one-letter and three-letter codes for naturally occurring amino acids, but also three-letter codes generally allowed for other amino acids, such as 2-aminoisobutyric acid (Aib), N-methylglycine (Sar), and α -methyl-glutamic acid, are used. The amino acids mentioned herein are abbreviated according to the nomenclature rules of IUPAC-IUB as follows.
- [63]
- [64] alanine (Ala, A), arginine (Arg, R)
- [65] asparagine (Asn, N), aspartic acid (Asp, D)
- [66] cysteine (Cys, C), glutamic acid (Glu, E)
- [67] glutamine (Gln, Q), glycine (Gly, G)
- [68] histidine (His, H), isoleucine (Ile, I)
- [69] leucine (Leu, L), lysine (Lys, K)
- [70] methionine (Met, M), phenylalanine (Phe, F)
- [71] proline (Pro, P), serine (Ser, S)
- [72] threonine (Thr, T), tryptophan (Trp, W)
- [73] tyrosine (Tyr, Y), valine (Val, V)
- [74]
- [75] An aspect of the present invention provides a lyophilized formulation including a fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region.
- [76] Specifically, the present invention relates to a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a pharmaceutically effective amount of a fusion protein in which α -galactosidase A and an immunoglobulin Fc region are linked to each other; a buffer; and a sugar or sugar alcohol. The aqueous solution may further contain an amino acid, a non-ionic surfactant, a preservative, or a combination thereof, but is not limited thereto.
- [77]
- [78] As used herein, the term "lyophilized formulation" refers to a drug formulated by lyophilization, as a form of a pharmaceutical product. Specifically, the α -galactosidase A fusion protein may be lyophilized together with a substance, such as an excipient,

used to stabilize the fusion protein and thus is present in a solid state. A component contained in the lyophilized formulation, in addition to the α -galactosidase A fusion protein exhibiting pharmacological efficacy, may be interchangeably used with stabilizers. As used herein, the term "stabilizer" refers to a substance that stably maintains components of a formulation, such as an active ingredient, for a predetermined period of time.

[79]

[80] In the present invention, the lyophilized formulation is a concept that includes a lyophilized substance. The above lyophilized formulation is manufactured through a freeze-drying process in the form of a pre-lyophilized formulation containing a stabilizers for stabilizing the α -galactosidase A fusion protein and the α -galactosidase A fusion protein. In the present invention, the lyophilized formulation of the α -galactosidase A fusion protein may include a therapeutically effective amount of the α -galactosidase A fusion protein, and the therapeutically effective amount of the α -galactosidase A fusion protein may be contained in a single-use container or a multi-use container, but is not limited thereto. The lyophilized formulation of the present invention has a composition capable of stabilizing the α -galactosidase A fusion protein during the lyophilization process, and even while the lyophilized formulation is stored and then reconstituted, the stability of the formulation can be maintained and the formulation has a high recovery rate of the α -galactosidase A fusion protein. Furthermore, the lyophilized formulation of the present invention can maintain the stability even when the α -galactosidase A fusion protein is contained therein at a high concentration of 10 mg/mL to 40 mg/mL.

[81] The lyophilized formulation that includes the α -galactosidase A fusion protein according to the present invention is stored in a container, and when needed, may be reconstituted for administration to an individual.

[82] As used herein, the term "reconstitution" refers to a process of liquefying (dissolving) the lyophilized substance in a solid state such that the α -galactosidase A fusion protein can be administered. Particularly, the concentration of the α -galactosidase A fusion protein included in the lyophilized formulation of the present invention may be 1 mg/mL to 150 mg/mL, 10 mg/mL to 120 mg/mL, or 10 mg/mL to 100 mg/mL upon reconstitution, but is not limited thereto. The concentration of the pre-formulation during a lyophilization process may be different from that after reconstitution.

[83]

[84] The lyophilized formulation of the present invention includes stabilizers capable of stabilizing the structure of the fusion protein so that the pharmacological efficacy of the α -galactosidase A fusion protein can be maintained for a long time even during long-term storage. Such a lyophilized formulation of the present invention includes a

mixture obtained by lyophilizing an aqueous solution containing the α -galactosidase A fusion protein, a buffer, and a sugar or sugar alcohol.

[85]

[86] The α -galactosidase A fusion protein of the present invention is in a form in which α -galactosidase A is fused to an immunoglobulin Fc region. The α -galactosidase A fusion protein undergoes unfolding, in which the structure of the protein is deformed by various external factors (e.g., pH, temperature, osmosis, and presence or absence of stabilizers) during a lyophilization, reconstitution, or storage process, and thus causes a loss of enzymatic activity, resulting in a significant deterioration in the pharmacological efficacy of the formulation that includes α -galactosidase. Particularly, one of the two domains constituting α -galactosidase A is first unfolded (to form soluble aggregates) and the other domain of α -galactosidase A and the immunoglobulin Fc region are sequentially unfolded, followed by the formation of higher-order aggregates and, finally, CH2 and CH3 domains of the immunoglobulin Fc region are unfolded (to form insoluble aggregates), resulting in precipitates visible to the naked eye. If the structure of the α -galactosidase A fusion protein is not maintained due to the stress occurring during the storage of the lyophilized formulation, resulting in soluble aggregates or higher-order aggregates, the risk of immunogenicity increases, causing safety problems with the formulation. Therefore, it is important to include a composition for preventing the unfolding of the α -galactosidase A fusion protein, in order to ensure the stability of the lyophilized formulation.

[87] The present inventors established that the pH conditions of a formulation and the inclusion or exclusion of sugars (a sugar or sugar alcohol) are important in order to increase the recovery rate of a native form when a lyophilized formulation containing an α -galactosidase A fusion protein is reconstituted, and completed the present invention. The lyophilized formulation of the present invention can maintain the structure and activity of the fusion protein during long-term storage and achieves a high recovery rate, and thus is more effective in treating patients.

[88] A specific example of the lyophilized formulation according to the present invention may be a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region; a buffer; and a sugar or sugar alcohol, more specifically, a fusion protein at 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; and a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL.

[89] Another example may be a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; a sugar or sugar alcohol at a

concentration of 1 to 20 mg/mL; and an amino acid at a concentration of 1.0 to 3.0% (w/v).

[90] Still another example may be a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; and a non-ionic surfactant at a concentration of 0.001 to 0.1% (w/v).

[91] Still another example may be a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; an amino acid at a concentration of 1.0 to 3.0% (w/v); and a non-ionic surfactant at a concentration of 0.001 to 0.1% (w/v).

[92] Still another example may be a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; mannitol, sucrose, or trehalose at a concentration of 1 to 20 mg/mL; serine at a concentration of 1.0 to 3.0% (w/v); and polysorbate 20 at a concentration of 0.001 to 0.1% (w/v).

[93] Still another example may be a lyophilized formulation including a mixture obtained by lyophilizing an aqueous solution containing: a fusion protein at a concentration of 15 to 35 mg/mL; histidine at a concentration of 5 to 30 mM; a sugar or sugar alcohol at a concentration of 2.5 to 10 mg/mL; polysorbate 20 at a concentration of 0.001 to 0.05% (w/v); and serine at a concentration of 1.0 to 3.0% (w/v), but the lyophilized formulation of the present invention is not limited to the above examples.

[94]

[95] As used herein, the term "aqueous solution" refers to a substance capable of stably storing the α -galactosidase A fusion protein and maintaining the stability during lyophilization and reconstitution while containing the α -galactosidase A fusion protein. Particularly, the aqueous solution contains an excipient for stabilizing the α -galactosidase A fusion protein, thereby imparting stability to the α -galactosidase A fusion protein during lyophilization and enabling the preparation of a lyophilized formulation with storage stability. The stabilizers may include a buffer and sugars (a sugar or sugar alcohol). The stabilizers may further include, but are not limited to, an amino acid, a non-ionic surfactant, a preservative, an antioxidant, and the like. In proteins such as the α -galactosidase A fusion protein, storage stability is important not only for accurate dosage but also for inhibiting the potential production of an antigenic substance against the α -galactosidase A fusion protein. The aqueous solution may be used interchangeably with "pre-formulation" in the present invention. The lyophilized formulation of the present invention comprises a lyophilized mixture of the aqueous

solution comprising α -galactosidase A fusion protein and stabilizers (e.g., buffer, sugar, sugar alcohol, amino acid, etc.)

[96]

[97] The concentration of the α -galactosidase A fusion protein may be controlled by adjusting the volume of a reconstitution solution added to the lyophilized formulation, and therefore, the concentration of the α -galactosidase A fusion protein in the aqueous solution is not particularly limited, but the aqueous solution can stably lyophilize the α -galactosidase A fusion protein at a high concentration of about 10 mg/mL or more, specifically about 10 mg/mL to 40 mg/mL, and can also maintain the stability of the α -galactosidase A fusion protein in a reconstituted solution.

[98]

For example, the aqueous solution of the present invention may contain the α -galactosidase A fusion protein at a concentration of about 10 to 90 mg/mL, about 10 to 70 mg/mL, about 10 to 50 mg/mL, about 10 to 30 mg/mL, about 15 to 50 mg/mL, about 15 to 40 mg/mL, about 15 to 30 mg/mL, about 20 to 30 mg/mL, about 10 mg/mL, 20 mg/mL, 22 mg/mL, or 30 mg/mL, but is not limited thereto.

[99]

As used herein, the term "about" refers to a range encompassing ± 0.5 , ± 0.4 , ± 0.3 , ± 0.2 , ± 0.1 , ± 0.01 , and the like, and includes all of the values in the range equivalent or similar to those stated after this term, but is not limited thereto.

[100]

It is generally known that increasing the protein concentration in a formulation may reduce stability and may cause the protein to precipitate. However, the aqueous solution containing stabilizers according to the present invention, even when containing the fusion protein at a high concentration, can maintain the structure of the fusion protein during lyophilization and reconstitution, suppress the occurrence of precipitation, and achieve a high recovery rate upon reconstitution. Therefore, the aqueous solution of the present invention may contain, as an active ingredient, the α -galactosidase A fusion protein at a high concentration, for example, at a concentration of about 10 mg/mL or more, about 15 mg/mL or more, or about 20 mg/mL or more.

[101]

[102]

Buffer, as a component contained in the aqueous solution of the present invention, is a solution that serves to maintain the pH of the aqueous solution to avoid a rapid change in pH of the formulation during lyophilization or after reconstitution. The aqueous solution may be used as a solvent for the α -galactosidase A fusion protein and the stabilizers, and any aqueous solution may be used as the buffer of the present invention without limitation as long as the aqueous solution can maintain the pH level at which the α -galactosidase A fusion protein is stabilized.

[103]

The pH value of the aqueous solution affects the structure of the fusion protein, and the structure of the fusion protein may be stably maintained at a pH of 6.5 to 8.0. If the

pH falls outside the appropriate range, α -galactosidase A is unfolded earlier than the immunoglobulin Fc region, resulting in impaired structural stability. Therefore, it is important to maintain the pH at an appropriate level to ensure structural stability of the α -galactosidase A fusion protein.

- [104] Particularly, the present inventors identified that when a lyophilized formulation prepared from an aqueous solution with a pH of 6.5 or higher was dissolved, the appearance was transparent even when refrigerated, and the recovery rate of a native form was also high.
- [105] Examples of the buffer may include phosphoric acid and its conjugate alkaline salts (e.g., a phosphate, such as sodium phosphate, potassium phosphate, or hydrogen or dihydrogen salts thereof), citric acid and its salts (e.g., sodium citrate), acetic acid and its salts (e.g., sodium acetate), histidine and its salts, and mixtures of these as buffers, but are not limited thereto. For example, the buffer may be selected from the group consisting of a citrate buffer (e.g., sodium citrate buffer), an acetate buffer (e.g., sodium acetate buffer), a phosphate buffer (e.g., sodium phosphate buffer), a histidine buffer, and a combination thereof, and the buffer or buffering substances in the aqueous solution (citric acid and a salt thereof, acetic acid and a salt thereof, histidine and a salt thereof, phosphoric acid and a salt thereof, or a combination thereof) may be contained at a concentration sufficient to maintain the pH for structural stability of proteins.
- [106] The pH of the aqueous solution may be about 6.5 to 8.0, for example, about pH 6.5 to 7.8, about pH 6.5 to 7.5, about pH 6.5 to 7.0, about pH 6.5 to 6.8, or a pH of about 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, or 7.5, but is not particularly limited thereto.
- [107] To achieve the target pH, the buffer may include phosphoric acid, citric acid, acetic acid, histidine, or a salt thereof, or a mixture thereof at a concentration of about 5 mM to about 200 mM, about 5 mM to about 100 mM, about 5 mM to about 80 mM, about 5 mM to about 40 mM, about 8 mM to about 40 mM, about 5 mM to about 30 mM, about 5 mM to about 25 mM, about 5 mM to about 15 mM, about 5 mM to about 10 mM, or about 6.67 mM, but is not particularly limited thereto.
- [108]
- [109] In a specific embodiment, the buffer may include about 10 mM histidine, and have a pH of about 6.5 or higher, specifically, about pH 6.5 to 8.0, about pH 6.5 to 7.5, about pH 6.5 to 7.0, or about pH 6.5 to 6.8, but is not limited thereto.
- [110]
- [111] The sugars (a sugar or sugar alcohol) contained in the aqueous solution of the present invention play a crucial role in increasing the recovery rate of α -galactosidase A fusion protein when a lyophilized mixture is dissolved. The present inventors identified that the recovery rate of the native α -galactosidase A fusion protein was high regardless

of the type of sugar in formulations including sugars under all pH conditions.

Therefore, the lyophilized formulation of the present invention, which achieves a high recovery rate of a native form by containing a sugar or sugar alcohol, can enhance the therapeutic effect by allowing the administration of the α -galactosidase A fusion protein at an appropriate dose.

- [112] The sugar refers to a monosaccharide, a disaccharide, a polysaccharide, or an oligosaccharide and examples thereof may be mannose, glucose, fructose, galactose, fucose, lactose, maltose, sucrose, trehalose, raffinose, dextran, or a combination thereof. In a specific embodiment, the sugar may be glucose, fructose, galactose, lactose, maltose, sucrose, trehalose, or a combination thereof, but is not limited thereto. For example, the sugar may be sucrose or trehalose, but is not particularly limited thereto.
- [113]
- [114] The sugar alcohol refers to a substance containing a plurality of hydroxyl groups, and includes a substance in which an aldehyde group and/or ketone group of a sugar is substituted with an alcohol group, and includes saccharides containing multiple hydroxyl groups. For example, the sugar alcohol may be mannitol, sorbitol, or a combination thereof, and specifically may be mannitol, but is not limited thereto.
- [115] The aqueous solution according to the present invention may contain one type of sugar, that is, a sugar or sugar alcohol, or may contain a combination of several types of sugars, but is not limited thereto.
- [116] The sugar alcohol, sugar, or a combination thereof may be present in the aqueous solution, at a concentration of about 1 to 30 mg/mL, about 1 to 25 mg/mL, about 2 to 20 mg/mL, about 2 to 15 mg/mL, about 2 to 10 mg/mL, about 2.5 to 5 mg/mL, or about 2.5 mg/mL, but is not particularly limited thereto.
- [117]
- [118] The aqueous solution of the present invention may further contain an amino acid. The amino acid may impart structural stability to the α -galactosidase A fusion protein. The amino acid contained in the lyophilized formulation of the present invention may be selected from serine, arginine, lysine, threonine, asparagine, glutamine, glycine, proline, alanine, valine, isoleucine, leucine, phenylalanine, or a combination thereof, and specifically, may be selected from the group consisting of serine, arginine, threonine, glutamine, glycine, alanine, and a combination thereof, but is not limited thereto. In a specific embodiment, the amino acid may be serine, but is not limited thereto.
- [119] Relative to the total volume of the aqueous solution of the present invention, the amino acid may be present in the aqueous solution at an amount of about 1.0 to 5.0% (w/v), about 1.0 to 4.0% (w/v), about 1.0 to 3.0% (w/v), about 1.4 to 3.0% (w/v), about

2.1 to 2.9% (w/v), about 1.4% (w/v), about 2.1% (w/v), about 2.5% (w/v), or about 2.9% (w/v), but is not particularly limited thereto.

[120]

[121] The aqueous solution according to the present invention may further contain a non-ionic surfactant. The non-ionic surfactant refers to a substance that lowers the surface tension of a protein solution to prevent the protein from being adsorbed to or aggregating on the hydrophobic surface after reconstitution.

[122] Specific examples of the non-ionic surfactant available in the present invention may include a polysorbate (e.g., polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate), polysorbate 40 (polyoxyethylene (20) sorbitan monopalmitate), polysorbate 60 (polyoxyethylene (20) sorbitan monostearate), polysorbate 80 (polyoxyethylene (20) sorbitan monooleate); wherein the number 20 following the polyoxyethylene indicates the total number of oxyethylene groups $-(\text{CH}_2\text{CH}_2\text{O})-$), poloxamer (PEO-PPO-PEO copolymer; wherein PEO: poly(ethylene oxide) and PPO: poly(propylene oxide)), polyethylene-polypropylene glycol, a polyoxyethylene compound (e.g., polyoxyethylene-stearate, polyoxyethylene alkyl ether (alkyl: C1-C30), polyoxyethylene monoallyl ether, alkylphenyl polyoxyethylene copolymer (alkyl: C1-C30), etc.), and sodium dodecyl sulphate (SDS), and the like or an example thereof may be polysorbate or poloxamer, and these substances may be used alone or in a combination of one or more thereof.

[123] Specifically, the non-ionic surfactant may be poloxamer 188, polysorbate 20, polysorbate 40, polysorbate 60, or polysorbate 80, which may be used in combination, but is not particularly limited thereto.

[124] In the present invention, the non-ionic surfactant may be contained in the aqueous solution of the present invention, relative to the total volume of the aqueous solution, at a concentration of about 0.1% (w/v) or less, for example, about 0.001 to about 0.1% (w/v), about 0.001 to about 0.1% (w/v), about 0.01 to about 0.05% (w/v), about 0.001% (w/v), about 0.0255% (w/v), or about 0.05% (w/v), but is not particularly limited thereto.

[125]

[126] The aqueous solution according to the present invention may not further contain an isotonic agent, and the isotonic agent may be sodium chloride, but is not limited thereto. The aqueous solution of the lyophilized formulation according to the present invention does not contain an isotonic agent and thus allows the collapse temperature during lyophilization to be set higher, thereby reducing the time for a lyophilization process and lowering the osmotic pressure. The present inventors identified that no collapse occurred when the lyophilized formulation containing no isotonic agent according to the present invention was prepared.

[127]

[128] The aqueous solution according to the present invention may further contain a preservative. The preservative is a substance that substantially mitigates the action of bacteria or fungi in the formulation and is a compound contained in the formulation to facilitate the production of multi-dose preparations. Examples of potential preservatives may include octadecyldimethyl-benzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chloride wherein an alkyl group is a long-chain compound), and benzethonium chloride. Other types of preservatives may include aromatic alcohols, such as phenol, butyl, or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol, but are not limited thereto. The concentration of the preservative may be 0.001% (w/v) to 1.0% (w/v), but is not limited thereto.

[129]

[130] Meanwhile, the aqueous solution (pre-formulation) for preparing the lyophilized formulation of the present invention may further optionally contain other components or substances known in the art, in addition to the above described buffer, sugar alcohol, sugar, amino acid, non-ionic surfactant, and/or preservative, within ranges that do not impair the advantageous effects of the present invention, but is not limited thereto.

[131]

[132] A specific example of the aqueous solution (pre-formulation) for preparing the lyophilized formulation according to the present invention may be an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; and a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL, but is not limited thereto.

[133]

Another specific example of the aqueous solution (pre-formulation) for preparing the lyophilized formulation according to the present invention may be an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; a non-ionic surfactant at a concentration of 0.001 to 0.1% (w/v); and an amino acid at a concentration of 1.0 to 3.0% (w/v), but is not limited thereto.

[134]

Another specific example of the aqueous solution (pre-formulation) for preparing the lyophilized formulation according to the present invention may be an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0 containing 5 to 30 mM histidine; a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL; polysorbate 20 at a concentration of 0.001 to 0.1% (w/v); and serine at a concentration of 1.0 to 3.0% (w/v), but is not limited thereto.

- [135] Another specific example of the aqueous solution (pre-formulation) for preparing the lyophilized formulation according to the present invention may be an aqueous solution containing: a fusion protein at a concentration of 10 to 40 mg/mL; a buffer with a pH of 6.5 to 8.0 containing 5 to 30 mM histidine; sucrose, trehalose, or mannitol at a concentration of 1 to 20 mg/mL; polysorbate 20 at a concentration of 0.001 to 0.1% (w/v); and serine at a concentration of 1.0 to 3.0% (w/v), but is not limited thereto.
- [136] Additionally, the aqueous solution for preparing the lyophilized formulation or the lyophilized formulation may further contain a pharmaceutically acceptable carrier, an excipient, and the like, and such a carrier or excipient may be non-naturally occurring.
- [137]
- [138] The lyophilized formulation according to the present invention may include a mixture obtained by lyophilizing 1 to 10 mL of an aqueous solution, and specifically, 5 to 10 mL or 4 to 6 mL of an aqueous solution, but is not limited thereto.
- [139] The present inventors prepared 4 or 6 mL of an aqueous solution, followed by lyophilization and subsequent dissolution, and as a result, identified that the recovery rate of a native form was higher for a charging amount of 6 mL, with no significant difference between the two charging amounts.
- [140]
- [141] The aqueous solution for preparing the lyophilized formulation of the present invention may be frozen and dried under appropriate freezing and drying conditions known in the art. The drying may be completed in a primary process or through several processes, such as secondary or higher processes.
- [142]
- [143] The lyophilized formulation of the present invention may be obtained by preparing a pre-formulation including the α -galactosidase A fusion protein at an appropriate concentration considering a desired dosage, lyophilizing the pre-formulation, and then again reconstituting the resultant product to be suitable for administration to a patient. The pre-formulation may be diluted to increase the volume, lyophilized, and then reconstituted with a smaller volume of reconstitution solution compared to the volume during reconstitution, but is not limited thereto. In the present invention, the lyophilized formulation may be a mixture obtained by lyophilizing an aqueous solution containing the α -galactosidase A fusion protein and stabilizers (e.g., a buffer, a sugar, a sugar alcohol, etc.), and further be a reconstituted formulation obtained by reconstituting the lyophilized formulation using a reconstitution solution, but is not limited thereto.
- [144]
- [145] The lyophilized formulation according to the present invention can maintain the physical and/or chemical stability of the α -galactosidase A fusion protein while

maintaining the activity thereof even during long-term storage, thereby ensuring excellent long-term stability. For example, the appearance, moisture content, and recovery rate of a native form can be maintained at appropriate levels even under long-term refrigerated conditions (5°C) or harsh conditions (40°C).

[146] Specifically, the lyophilized formulation according to the present invention can be stable for a period of time of 2 weeks or longer, 1 month or longer, 3 months or longer, 6 months or longer, 12 months or longer, 18 months or longer, or 24 months or longer even at a temperature of about -80°C to 50°C, for example, about 2°C to 8°C, about 5°C, about 35°C, about 40°C, 35°C or higher, or about 40°C or higher, but is not limited thereto.

[147]

[148] The stability of the lyophilized formulation may be examined by a method known in the art, including pharmacopoeia specifications, and test items and criteria for assessing stability may be appropriately set by a person skilled in the art, and the examination is not limited to specific methods.

[149] For example, the lyophilized formulation according to the present invention remains transparent in terms of appearance as a cake or appearance after dissolution even during long-term storage, has a moisture content of less than 5%, less than 4%, less than 3%, less than 2%, or less than 1%, resulting in no deterioration in product quality, and shows a recovery rate of a native form of 85% or more, 90% or more, 95% or more, or 97% or more in SE-HPLC, but is not limited thereto.

[150]

[151] Hereinafter, the α -galactosidase A fusion protein as an active ingredient contained in the lyophilized formulation of the present invention will be described in more detail.

[152]

[153] The α -galactosidase A fusion protein contained in the lyophilized formulation of the present invention refers to a fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region, and the fusion protein may have a form in which two molecules of α -galactosidase A are linked to the immunoglobulin Fc region in a dimeric form via linkers. Specifically, two molecules of α -galactosidase A may be linked to respective monomers of the immunoglobulin Fc region in a dimeric form via linkers, respectively. The two molecules of α -galactosidase A may form a dimer through non-covalent bonds, but are not limited thereto. The fusion protein of the present invention has increased stability of α -galactosidase A due to the fusion of the immunoglobulin Fc region to the α -galactosidase A thereby exerting pharmacological efficacy for a long time in the body. The lyophilized formulation of the present invention does not lose the pharmacological efficacy despite long-term storage since a

pre-formulation containing stabilizers in addition to the fusion protein is lyophilized to impart excellent stability to the fusion protein.

[154] Specifically, the fusion protein of the present invention may include the amino acid sequence of SEQ ID NO: 4, or may be encoded by the polynucleotide sequence of SEQ ID NO: 5, but is not limited thereto. The fusion protein of the present invention may be a dimer formed of two monomers including the amino acid sequence of SEQ ID NO: 4, but is not limited thereto. For the fusion protein of the present invention, the disclosure of WO 2019/125059 is incorporated as a reference.

[155]

[156] As used herein, the terms "fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region", " α -galactosidase A fusion protein", and "fusion protein" may be interchangeably used.

[157] The fusion protein of the present invention is expressed within a transformant in a form in which α -galactosidase A is linked to the immunoglobulin Fc region via a linker, so that α -galactosidase A forms a dimer via non-covalent bonds when the immunoglobulin Fc region forms the dimer.

[158]

[159] The α -galactosidase A (alpha-galactosidase A, α -Gal A, GLA) of the present invention is an enzyme that is present in lysosomes of the spleen, brain, liver, and the like and hydrolyzes α -galactosyl moieties at the ends of glycolipids and glycoproteins, and is a homodimeric glycoprotein. Particularly, α -galactosidase A is known to be involved in Fabry disease, which is a lysosomal storage disease. The α -galactosidase A has a dimeric structure consisting of two domains (TIM barrel domain and β -sheet containing immunoglobulin-like domain) (Journal of Biological Chemistry, Vol. 287, No. 34, 2012; and Lieberman et al. Biochemistry, Vol. 48, No. 22, 2009). The α -galactosidase A unfolds under a high pH environment, and therefore a formulation containing α -galactosidase A is required to maintain an appropriate level of pH. Particularly, the active site is maintained in the state of soluble aggregates formed as one domain unfolds, but the activity is lost once insoluble aggregates are formed as unfolding further proceeds, and thus the lyophilized formulation is required to include stabilizers to exert the enzymatic activity by maintaining the soluble aggregate state. In the present invention, the α -galactosidase A may be in a native form or in a recombinant form and, specifically, may include the amino acid sequence of SEQ ID NO: 1, but is not limited thereto.

[160] The α -galactosidase A includes fragments of a native form, or analogs thereof, without limitation, having mutations selected from the group consisting of substitution, addition, deletion, modification, and a combination thereof as long as the analogs have activity equivalent to that of the native form of the enzyme.

- [161] The α -galactosidase A may include an amino acid sequence having at least 60%, 70%, or 80%, specifically at least 90%, more specifically, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% homology or identity with the amino acid sequence of SEQ ID NO: 1, and may be obtained from microorganisms by using recombination technology or be commercially available, but is not limited thereto.
- [162] As used herein, the term "homology" refers to a degree of similarity with a wild-type amino acid sequence or a wild-type nucleotide sequence, and the homology comparison may be conducted by observation with the naked eye or using a commercially available comparison program. The commercially available computer program may be used to calculate the homology between two or more sequences as a percentage (%). The homology (%) may be calculated between adjacent sequences. The terms "homology" and "identity" may often be used interchangeably.
- [163] The information on the sequences of α -galactosidase A or derivatives thereof and nucleotide sequences encoding the same may be obtained from known databases, such as NCBI.
- [164] The α -galactosidase A fusion protein of the present invention may be prepared by expression in the form, in which α -galactosidase A is linked to an immunoglobulin Fc region via a linker, within a transformant.
- [165]
- [166] The linker is a peptide linker, and the fusion protein of the present invention may be in the form in which α -galactosidase A is linked to the immunoglobulin Fc region via the peptide linker. One end of the linker may be linked to one chain of the immunoglobulin Fc region in a dimer form, but is not limited thereto.
- [167]
- [168] The peptide linker may contain one or more amino acids, for example, 1 to 1000 amino acids. The peptide linker may be any peptide linker known in the art, for example, including [GS] x linker, [GGGS] x linker, and [GGGGS] x linker, and the like, wherein x may be a natural number of 1 or greater (e.g., 1, 2, 3, 4, 5, or greater), but is not limited thereto. Specifically, the peptide linker of the present invention may be composed of 10 to 50 amino acids, more specifically, 20 to 40 amino acids, and may include the amino acid sequence of SEQ ID NO: 2.
- [169]
- [170] In an aspect of the present invention, the sites of the α -galactosidase A and the immunoglobulin Fc region, to which the peptide linker is linked, are not limited as long as the activity of α -galactosidase A is maintained, and α -galactosidase A is linked to the immunoglobulin Fc region. Specifically, the sites may be at a terminal of each of the α -galactosidase A and the immunoglobulin Fc region, more specifically, the

C-terminus of the α -galactosidase A and the N-terminus of the immunoglobulin Fc region, but are not limited thereto.

[171] As used herein, the term "N-terminus" or "C-terminus" refers to the amino terminus and the carboxyl terminus of a protein, respectively. Examples thereof may include the last amino acid residue of the N-terminus or the C-terminus but also amino acid residues near the N-terminus or the C-terminus, specifically, the first amino acid residue to the 10th amino acid residue from the last end.

[172] In the present invention, the peptide linkers may be linked to the monomers of the immunoglobulin Fc region in a dimeric form composed of monomeric immunoglobulin Fc regions, respectively, and each of the linkers respectively linked to the monomers of the immunoglobulin Fc region may be independently linked to one molecule of α -galactosidase A, respectively, but are not limited thereto.

[173]

[174] The immunoglobulin Fc region, one moiety constituting the enzyme fusion protein of the present invention, may be a dimer formed of immunoglobulin Fc region monomers.

[175] As used herein, the term "immunoglobulin Fc region" refers to a region including a heavy chain constant domain 2 (CH2) and/or a heavy chain constant domain 3 (CH3) excluding the heavy chain and light chain variable domains of immunoglobulin. In an aspect of the present invention, such an immunoglobulin Fc region may include a modified hinge region, but is not limited thereto. Specifically, the immunoglobulin Fc region may have mutations selected from the group consisting of substitution, addition, deletion, modification, and any combination thereof in the native immunoglobulin Fc region, but is not limited thereto.

[176] The immunoglobulin Fc region is a substance used as a carrier in drug manufacturing. To stabilize proteins and prevent the elimination of proteins by the kidneys, research on fusion proteins using immunoglobulin Fc regions has recently been actively conducted. Immunoglobulins are major constituents of the blood, and there are five different types, IgG, IgM, IgA, IgD, and IgE. The most frequently used type for fusion protein studies is IgG, which is classified into four subtypes, IgG1 to IgG4.

[177] The immunoglobulin Fc region may include a hinge region in the heavy chain constant domain, and the immunoglobulin Fc region monomers may constitute a dimer via the hinge region, but is not limited thereto. The immunoglobulin Fc region of the present invention may be an extended Fc region including a part of or the entirety of a heavy chain constant domain 1 (CH1) and/or a light chain constant domain 1 (CL1) excluding the heavy chain and the light chain variable domains of the immunoglobulin, as long as the immunoglobulin Fc region has substantially identical or enhanced effects compared with the native form. Additionally, the immunoglobulin Fc region

of the present invention may be a region from which a considerably long amino acid sequence corresponding to CH2 and/or CH3 is eliminated.

[178] Specifically, the immunoglobulin Fc region of the present invention may include 1) a CH1 domain, a CH2 domain, a CH3 domain, and a CH4 domain, 2) a CH1 domain and a CH2 domain, 3) a CH1 domain and a CH3 domain, 4) a CH2 domain and a CH3 domain, or 5) a combination of one or more domains selected from a CH1 domain, a CH2 domain, a CH3 domain, and a CH4 domain and an immunoglobulin hinge region (or a part of the hinge region), but is not limited thereto. However, the immunoglobulin Fc region of the present invention is not limited thereto. More specifically, the immunoglobulin Fc region may include a hinge region, a CH2 domain, and a CH3 domain, but is not limited thereto.

[179]

[180] As used herein, the term "hinge sequence" refers to a site located on a heavy chain to form the dimer of the immunoglobulin Fc region via an inter-disulfide bond, and the immunoglobulin Fc region of the present invention may be in a form in which two molecules of immunoglobulin Fc chains form a dimer due to the presence of the hinge sequence.

[181] Specifically, the hinge region may be mutated to include only one cysteine (Cys) by a deletion of a part of the hinge region, or to include a proline (Pro) residue substituted for a serine (Ser) residue involved in chain exchange. More specifically, the hinge region may have a substitution of the serine residue at the 2nd position with a proline residue, but is not limited thereto. The immunoglobulin Fc region of the present invention may include the amino acid sequence of SEQ ID NO: 3, but is not limited thereto.

[182]

[183] The immunoglobulin Fc region of the present invention encompasses not only a native sequence obtained from papain digestion of an immunoglobulin, but also derivatives, substituents, and variants thereof, for example, sequences different from the native sequence and obtained by mutation of one or more amino acid residues by deletion, addition, non-conservative or conservative substitution, or a combination thereof. The derivatives, substituents, and variants are assumed to possess the ability to bind to FcRn.

[184] For example, the amino acid residues at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331 in IgG Fc, which are known to be important for linkage, may be used as sites suitable for mutation.

[185] In addition, various types of derivatives are possible by, for example, removing a site capable of forming a disulfide bond, deleting some amino acid residues at the N-terminus of native Fc, or adding a methionine residue at the N-terminus of

native Fc. In addition, for removal of effector functions, complement-binding sites, for example, a C1q-binding site, may be removed, and an antibody dependent cell mediated cytotoxicity (ADCC) site may be removed. The techniques for preparing such sequence derivatives of the immunoglobulin Fc region are disclosed in WO 97/34631 and WO 96/32478, and the like.

- [186] Amino acid exchanges in proteins and peptides that do not alter the entire activity of molecules are well known in the art (H. Neurath, R. L. Hill, *The Proteins*, Academic Press, New York, 1979). The exchanges that occur most commonly are exchanges between amino acid residues Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly. In some cases, modifications may occur by phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, amidation, or the like.
- [187] The above described Fc derivatives exhibit a biological activity equivalent to that of the Fc region of the present invention, and may have an enhanced structural stability of the Fc region against heat, pH, and the like.
- [188] Such an Fc region may be obtained from native forms isolated from living bodies of humans or animals, such as cows, goats, pigs, mice, rabbits, hamsters, rats, and guinea pigs, or may be recombinant forms or derivatives thereof obtained from transformant animal cells or microorganisms. Particularly, the Fc region may be obtained from a native form by isolating a whole immunoglobulin from a living human or animal body and then treating the isolated immunoglobulin with protease. The isolated whole immunoglobulin is digested into Fab and Fc when treated with papain, and digested into pF'c and F(ab)₂ when treated with pepsin. These fragments may be subjected to size exclusion chromatography or the like to separate Fc or pF'c therefrom. In a more specific embodiment, the immunoglobulin Fc region is a recombinant immunoglobulin Fc region where a human-derived Fc region is obtained from a microorganism.
- [189] Alternatively, the immunoglobulin Fc region may be in the form of native glycans, increased glycans compared with the native form, or decreased glycans compared with the native form, or may be in a deglycosylated form. The increase, decrease, or removal of the immunoglobulin Fc glycans may be achieved by using conventional methods, such as a chemical method, an enzymatic method, and a genetic engineering method using a microorganism. Particularly, the immunoglobulin Fc region obtained by removal of glycans from Fc exhibits a significant deterioration in binding affinity for the complement c1q and a reduction or loss in antibody-dependent cytotoxicity or complement-dependent cytotoxicity, and thus causes no unnecessary immune response in vivo. In this regard, a deglycosylated or aglycosylated immunoglobulin Fc region may be more suitable as a drug carrier for its own purpose.

- [190] As used herein, the term "deglycosylation" indicates an Fc region in which glycans are removed by an enzyme, and the term aglycosylation indicates a non-glycosylated Fc region produced in prokaryotes, more specifically *E. coli*.
- [191]
- [192] Meanwhile, the immunoglobulin Fc region of the present invention may originate from humans, or animals, such as cows, goats, pigs, mice, rabbits, hamsters, rats, and guinea pigs, and more specifically, the immunoglobulin Fc region originates from humans.
- [193] In addition, the immunoglobulin Fc region may be an Fc region derived from IgG, IgA, IgD, IgE, IgM, or a combination or hybrid thereof. In a more specific embodiment, the immunoglobulin Fc region is derived from IgG or IgM, which is most abundant in the human blood, and in a still more specific embodiment, the immunoglobulin Fc region is derived from IgG, which is known to increase the half-lives of ligand-binding proteins. In a still more specific embodiment, the immunoglobulin Fc region is an IgG4 Fc region, and in a still more specific embodiment, the immunoglobulin Fc region is an aglycosylated Fc region derived from human IgG4, but is not limited thereto. The immunoglobulin Fc region of the present invention may include the amino acid sequence of SEQ ID NO: 3, but is not limited thereto. More specifically, the immunoglobulin Fc region may include a monomer having the amino acid sequence of SEQ ID NO: 3, and the immunoglobulin Fc region may be a homodimer of monomers each having the amino acid sequence of SEQ ID NO: 3, but is not limited thereto.
- [194]
- [195] As used herein, the term "combination" refers to the formation of a linkage between a polypeptide encoding a single-chain immunoglobulin Fc region of the same origin and a single-chain polypeptide of a different origin when a dimer or a multimer is formed. That is, a dimer or multimer may be prepared from two or more fragments selected from the group consisting of IgG Fc, IgA Fc, IgM Fc, IgD Fc, and IgE Fc fragments.
- [196]
- [197] The immunoglobulin Fc region of the α -galactosidase A fusion protein may be in a dimeric form, specifically, and may have a structure in which two polypeptide chains are linked to each other via a disulfide bond. More specifically, the two chains may be linked via a nitrogen atom of one chain thereof, but are not limited thereto. The linkage via a nitrogen atom may be achieved by reductive amination of an ϵ -amino atom of lysine or the N-terminal amino group, but is not limited thereto. In one specific embodiment, the immunoglobulin Fc region may achieve the linkage via a nitrogen atom of proline at the N-terminus thereof, but is not limited thereto. One Fc region of

the dimeric form may be covalently linked to two molecules of α -galactosidase A via two linkers, but is not limited thereto.

[198]

[199] Unless otherwise stated herein, the disclosure of the α -galactosidase A or fusion protein according to the present invention in the detailed description or claims may be applied to not only the α -galactosidase A or fusion protein but also salts thereof (e.g., pharmaceutically acceptable salts) or solvate forms thereof. Thus, although only " α -galactosidase A" or "fusion protein" is described herein, the corresponding disclosure may also be applied to particular salts thereof, particular solvates thereof, and solvates of the particular salts. These salts may be, for example, in the form in which any pharmaceutically acceptable salt is used. The type of salts is not particularly limited. However, the salts are preferably in the form that is safe and effective for a subject, for example, a mammal, but is not particularly limited thereto.

[200] The term "pharmaceutically acceptable" refers to a substance that can be effectively used for a desired purpose without causing excessive toxicity, irritation, allergic responses, and the like within the scope of the medical and pharmaceutical decision.

[201] As used herein, the term "pharmaceutically acceptable salt" refers to a salt derived from pharmaceutically acceptable inorganic acids, organic acids, or bases. Examples of suitable salts may include hydrochloric acid, bromic acid, sulfuric acid, nitric acid, perchloric acid, fumaric acid, maleic acid, phosphoric acid, glycolic acid, lactic acid, salicylic acid, succinic acid, toluene-p-sulfonic acid, tartaric acid, acetic acid, citric acid, methanesulfonic acid, formic acid, benzoic acid, malonic acid, naphthalene-2-sulfonic acid, benzene sulfonic acid, and the like. Salts derived from suitable bases may include alkali metals such as sodium and potassium, alkali earth metals such as magnesium, ammonium, and the like.

[202] As used herein, the term "solvate" refers to a complex formed between the α -galactosidase A, fusion protein, or salt thereof according to the present invention and a solvent molecule.

[203]

[204] The fusion protein may be prepared or manufactured by any method known in the art, and specifically, the fusion protein may be obtained by culturing animal cells, into which an expression vector is inserted, and purifying the culture, or may be synthesized on the basis of the sequence thereof, but is not limited thereto.

[205]

[206] The lyophilized formulation of the present invention may be used to prevent, treat, and alleviate a disease, such as α -galactosidase A deficiency, which may be prevented, treated, or alleviated by administration of the α -galactosidase A fusion protein, but is not limited thereto.

- [207] The α -galactosidase A fusion protein of the present invention may be used as a drug for Enzyme Replacement Therapy (ERT). The Enzyme Replacement Therapy enables the prevention or treatment of a disease through the recovery of enzyme hypofunction by supplementing deficient or insufficient enzymes causing the disease.
- [208] Specifically, the lyophilized formulation of the present invention may be used to prevent, treat, or alleviate α -galactosidase A deficiency. The α -galactosidase A deficiency is a lysosomal storage disease caused by deficiency of α -galactosidase A (α -Gal A), a lysosomal enzyme, and examples thereof include Fabry Disease, Angiokeratoma Diffuse, Angiokeratoma Corporis Diffusum, and Hereditary Dystopic Lipidosis.
- [209] Fabry disease, one of the lysosomal storage diseases, is a recessively inherited disorder caused by X-chromosomal inactivation. Fabry disease is a congenital metabolic disorder of glycosphingolipid caused by deficient or insufficient activity of α -galactosidase A. It has been known that globotriaosylceramide (Gb3) is abnormally accumulated on the blood vessel wall and various parts of the body, such as skin, kidneys, heart, and nervous system, due to the abnormality of α -galactosidase A, significantly affecting the reduction of blood circulation and nutrient supply. Symptoms, such as hypersensitivity to cold, severe pain, acroparesthesia, angiokeratoma, corneal opacity, cardiac ischemia, myocardial infarction, and renal failure, occur, and eventually the kidneys fail to function properly, resulting in death.
- [210] As used herein, the term "prevention" refers to any action that inhibits or delays the onset of a disease by administration of the fusion protein or a lyophilized formulation containing the fusion protein, and the term "treatment" refers to any action that alleviates, ameliorates, or beneficially changes the symptoms of a target disease by administration of the fusion protein or a lyophilized formulation containing the fusion protein.
- [211]
- [212] In a specific embodiment of the present invention, the lyophilized formulation of the present invention may be used to prevent or treat Fabry disease, but is not limited thereto.
- [213] In addition, the lyophilized formulation of the present invention may be administered via a subcutaneous route, but is not limited thereto. Specifically, the lyophilized formulation of the present invention in a lyophilized state may be reconstituted to be suitable for subcutaneous administration.
- [214] As used herein, the term "administration" refers to the introduction of the reconstituted lyophilized formulation into a patient by any suitable method. The administration route of the lyophilized formulation is not particularly limited, but the lyophilized formulation may be administered by any general route as long as the α -

galactosidase A fusion protein of the lyophilized formulation can reach a target in the living body, for example, intraperitoneal administration, intravenous administration, intramuscular administration, subcutaneous administration, intradermal administration, oral administration, topical administration, intranasal administration, intrapulmonary administration, or rectal administration, but is not limited thereto.

[215]

[216] A preferred dosage of the fusion protein of the present invention may be about 0.0001 mg to 500 mg per 1 kg of the body weight of a patient per day, and the lyophilized formulation of the present invention may be formulated for appropriate dosage administration or reconstituted for appropriate dosage administration. However, with respect to the dosage of the fusion protein, the effective dose thereof is determined considering various factors, such as the patient's age, body weight, health condition, and sex, the severity of the disease, diet, and excretion rate, in addition to the route of administration and the frequency of treatment, and therefore, considering these, a person skilled in the art can easily determine an effective dose that is appropriate for the particular use of the lyophilized formulation of the present invention.

[217]

[218] In accordance with still another aspect of the present invention, there is provided a method for preparing the lyophilized formulation.

[219] Specifically, the preparation method may include lyophilizing an aqueous solution containing an α -galactosidase A fusion protein, a buffer, and a sugar or sugar alcohol.

[220] The aqueous solution or lyophilized formulation may further contain at least one component selected from the group consisting of an amino acid, a non-ionic surfactant, and a preservative.

[221] The aqueous solution, lyophilized formulation, and constituting components thereof are as described above.

[222] The method may include lyophilizing 1 to 10 mL, specifically, 5 to 10 mL of an aqueous solution containing: an α -galactosidase A fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region; a buffer; and a sugar or sugar alcohol, but is not limited thereto.

[223]

[224] In accordance with still another aspect of the present invention, there is provided a method for reconstituting the lyophilized formulation, the method including adding a reconstitution solution to the lyophilized formulation.

[225] The aqueous solution, lyophilized formulation, and reconstitution are as described above.

- [226] As used herein, the term "reconstitution solution" refers to a solution that is added to a lyophilized formulation in a solid state to achieve reconstitution. Examples of the reconstitution solution may include distilled water (water), but are not particularly limited thereto.
- [227] The reconstituted formulation may contain an α -galactosidase A fusion protein at a concentration suitable for administration to a subject, and specifically, may contain an α -galactosidase A fusion protein at a concentration of 10 to 100 mg/mL, 10 to 90 mg/mL, 10 to 50 mg/mL, or 10 to 30 mg/mL, but is not limited thereto.
- [228] The reconstituted formulation may be obtained by reconstituting the lyophilized formulation according to the present invention to be suitable for subcutaneous administration to a subject, but is not limited thereto.
- [229]
- [230] In accordance with still another aspect of the present invention, there is provided a lyophilized formulation for preventing or treating a disease.
- [231] The lyophilized formulation is as described above.
- [232] An example of the disease may be Fabry disease, but any disease for which the lyophilized formulation or the α -galactosidase A fusion protein included therein in the present invention can exhibit a preventive or therapeutic effect may be included without limitation.
- [233]
- [234] In accordance with still another aspect of the present invention, there is provided the use of the lyophilized formulation for preventing or treating a disease.
- [235] The lyophilized formulation and disease are as described above.
- [236]
- [237] Hereinafter, the present invention will be described in detail with reference to examples and experimental examples. However, the following examples and experimental examples are only for illustrating the present invention, and the scope of the present invention is not limited thereto.
- [238]
- [239] **Example 1: Preparation of fusion protein of α -galactosidase A and immunoglobulin Fc region**
- [240]
- [241] A fusion protein of α -galactosidase A and an immunoglobulin Fc region (hereinafter, referred to as α -galactosidase A fusion protein, SEQ ID NO: 4), in which native α -galactosidase A (SEQ ID NO: 1) and an immunoglobulin Fc region (SEQ ID NO: 3) were linked via a linker (SEQ ID NO: 2), was prepared.
- [242]

- [243] Specifically, a polynucleotide (SEQ ID NO: 5) encoding the α -galactosidase A fusion protein was inserted into an XOGC vector as an expression vector, by using a restriction enzyme to construct a vector expressing the α -galactosidase A fusion protein.
- [244]
- [245] The DNA and protein sequences of the α -galactosidase A fusion protein are as shown in Table 1 below. In the protein sequence of Table 1 below, the underlined texts indicate a signal sequence, bold letters indicate amino acid substitutions, and italic letters indicate the linker. The α -galactosidase A fusion protein of the present invention is a dimer formed of two monomers each including the amino acid sequence of SEQ ID NO: 4.
- [246]
- [247] [Table 1]

	Sequence			SEQ ID NO
Protein	MQLRNPELHL	GCALALRFLA	LVSWDIPGAR	4
	ALDNGLARTP	TMGWLHWERF	MCNLDCQEEP	
	DSCISEKLFM	EMAELMVSEG	WKDAGYEYLC	
	IDDCWMAQR	DSEGRLOADP	QRFPHGIRQL	
	ANYVHSKGLK	LGIYADVGNK	TCAGFPGSFG	
	YYDIDAQTFA	DWGVDLLKFD	GCYCDSLENL	
	ADGYKHMSLA	LNRTGRSIVY	SCEWPLYMWP	
	FQKPNYTEIR	QYCNHWRNFA	DIDDSWKSIIK	
	SILDWTSFNQ	ERIVDVAGPG	GWNDPDMLVI	
	GNFGLSWNQQ	VTQMALWAIM	AAPLFMSNDL	
	RHISPOAKAL	LQDKDVIIN	QDPLGKQGYQ	
	LRQGDNFEVW	ERPLSGLAWA	VAMINRQEIG	
	GPRSYTIAVA	SLGKGVACNP	ACFITQLLPV	
	KRKLGFYEW	SRLRSHINPT	GTVLLQLENT	
	MQMSLKDLLG	GGGSGGGGSG	GGGSGGGGSG	
	GGGSGGGGSP	PCPAPEFLGG	PSVFLFPPKP	
	KDTLMISRTP	EVTCVVVDVS	QEDPEVQFNW	
	YVDGVEVHNA	KTKPREEQFQ	STYRVVSVLT	
	VLHQDWLNGK	EYKCKVSNKG	LPSSIEKTIS	
	KAKGQPREPQ	VYTLPPSQEE	MTKNQVSLTC	
	LVKGFYPSDI	AVEWESNGQP	ENNYKTPPV	
	LDSDGSFFLY	SRLTVDKSRW	QEGNVFSCSV	
	MHEALHNHYT	QKSLSLSLGK		
DNA	ATGCAGCTGA	GGAACCCAGA	ACTACATCTG	5
	GGCTGCGCGC	TTGCGCTTCG	CTTCCTGGCC	
	CTCGTTTCCT	GGGACATCCC	TGGGGCTAGA	
	GCACTGGACA	ATGGATTGGC	AAGGACGCCT	
	ACCATGGGCT	GGCTGCACTG	GGAGCGCTTC	
	ATGTGCAACC	TTGACTGCCA	GGAAGAGCCA	
	GATTCCTGCA	TCAGTGAGAA	GCTCTTCATG	
	GAGATGGCAG	AGCTCATGGT	CTCAGAAGGC	
	TGGAAGGATG	CAGGTTATGA	GTACCTCTGC	
	ATTGATGACT	GTTGGATGGC	TCCCCAAAGA	
	GATTCAGAAG	GCAGACTTCA	GGCAGACCCT	
	CAGCGCTTTC	CTCATGGGAT	TCGCCAGCTA	
	GCTAATTATG	TTCACAGCAA	AGGACTGAAG	
	CTAGGGATTT	ATGCAGATGT	TGGAAATAAA	
	ACCTGCGCAG	GCTTCCCTGG	GAGTTTTGGA	
	TACTACGACA	TTGATGCCCA	GACCTTTGCT	
	GACTGGGGAG	TAGATCTGCT	AAAATTTGAT	

[248]

GGTTGTTACT	GTGACAGTTT	GGAAAATTTG
GCAGATGGTT	ATAAGCACAT	GTCCTTGGCC
CTGAATAGGA	CTGGCAGAAG	CATTGTGTAC
TCCTGTGAGT	GGCCTCTTTA	TATGTGGCCC
TTTCAAAAGC	CCAATTATAC	AGAAATCCGA
CAGTACTGCA	ATCACTGGCG	AAATTTTGCT
GACATTGATG	ATTCTTGGAA	AAGTATAAAG
AGTATCTTGG	ACTGGACATC	TTTTAACCAG
GAGAGAATTG	TTGATGTTGC	TGGACCAGGG
GGTTGGAATG	ACCCAGATAT	GTTAGTGATT
GGCAACTTTG	GCCTCAGCTG	GAATCAGCAA
GTAAGTCAGA	TGGCCCTCTG	GGCTATCATG
GCTGCTCCTT	TATTCATGTC	TAATGACCTC
CGACACATCA	GCCCTCAAGC	CAAAGCTCTC
CTTCAGGATA	AGGACGTAAT	TGCCATCAAT
CAGGACCCCT	TGGGCAAGCA	AGGGTACCAG
CTTAGACAGG	GAGACAACTT	TGAAGTGTGG
GAACGACCTC	TCTCAGGCTT	AGCCTGGGCT
GTAGCTATGA	TAAACCGGCA	GGAGATTGGT
GGACCTCGCT	CTTATACCAT	CGCAGTTGCT
TCCCTGGGTA	AAGGAGTGGC	CTGTAATCCT
GCCTGCTTCA	TCACACAGCT	CCTCCCTGTG
AAAAGGAAGC	TAGGGTTCTA	TGAATGGACT
TCAAGGTTAA	GAAGTCACAT	AAATCCCACA
GGCACTGTTT	TGCTTCAGCT	AGAAAATACA
ATGCAGATGT	CATTAAAAGA	CTTACTGGGC
GGCGGAGGTT	CAGGTGGTGG	TGGCTCTGGC
GGTGGAGGGT	CGGGGGGAGG	CGGCTCTGGA
GGAGGGGGCT	CCGGTGGGGG	AGGTAGCCCA
CCATGCCAG	CACCTGAGTT	CCTGGGGGGA
CCATCAGTCT	TCCTGTTCCC	CCCAAAACCC
AAGGACACCC	TCATGATCTC	CCGGACCCCT
GAGGTCACAT	GCGTGGTGGT	GGACGTGAGC
CAGGAAGACC	CTGAGGTCCA	GTTCAACTGG
TACGTGGACG	GCGTGGAGGT	GCATAATGCC
AAGACAAAGC	CGCGGGAGGA	GCAGTTCCAA
AGCACGTACC	GTGTGGTCAG	CGTCCTCACC
GTCCTGCACC	AGGACTGGCT	GAATGGCAAG
GAGTACAAGT	GCAAGGTCTC	CAACAAAGGC
CTCCCATCCT	CCATCGAGAA	AACCATCTCC
AAAGCCAAAG	GGCAGCCCCG	AGAACCACAG
GTGTACACCC	TGCCCCCATC	CCAGGAGGAG
ATGACCAAGA	ACCAGGTCAG	CCTGACCTGC
CTGGTCAAAG	GCTTCTATCC	CAGCGACATC

[249]		GCCGTGGAGT	GGGAGAGCAA	TGGGCAGCCG	
		GAGAACAAC	ACAAGACCAC	GCCTCCCGTG	
		CTGGACTCCG	ACGGCTCCTT	CTTCCTCTAC	
		AGCAGGCTAA	CCGTGGACAA	GAGCAGGTGG	
		CAGGAGGGGA	ACGTCTTCTC	ATGCTCCGTG	
		ATGCATGAGG	CTCTGCACAA	CCACTACACG	
		CAGAAGAGCC	TCTCCCTGTC	TCTGGGTAAA	TGA

[250]

[251] The constructed vector (pX0GC- α -galactosidase-Fc) expressing the α -galactosidase A fusion protein was transfected into a CHO-S cell line to prepare a cell line capable of mass producing the α -galactosidase A fusion protein.

[252]

[253] Specifically, CHO-S cells were suspension-cultured in a 1-L Erlenmeyer flask (Corning, cat. No. 431147) using a serum-free medium (FreeStyle CHO Expression Medium, Thermo Fisher, cat. No. 12651014). When the cells in the culture vessel reached 5×10^8 , the cells were transformed using FreeStyle Max (Thermo Fisher, cat. No. 16447-100). That is, 10 mL of OptiPro SFM (Thermo Fisher, cat. No. 12309-019) was placed into each of two tubes. To one tube, 500 μ g of DNA was added, and to the other tube, 500 μ L of FreeStyle Max was added. Then, the two solutions were mixed and allowed to stand at room temperature for 10 minutes. Thereafter, the mixture was added to the cells, for which the medium had been pre-exchanged with fresh FreeStyle CHO expression medium (Thermo Fisher, cat. No. 12651014). The cells were cultured for about 96 hours under the conditions of 37°C, 5% CO₂, and 125 rpm to prepare the α -galactosidase A fusion protein.

[254]

[255] In the α -galactosidase A fusion protein thus prepared, each of the two immunoglobulin Fc region monomers constituting the dimer is linked to one α -galactosidase A, thereby forming a structure in which the immunoglobulin Fc region in the dimeric form is fused to two molecules of α -galactosidase A.

[256]

[257] **Example 2: Improvement in appearances of lyophilized formulation according to pH changes**

[258]

[259] Example 2-1: Preparation of lyophilized formulations

[260]

[261] Several lyophilized formulations were prepared with various combinations of pH and contents of the nonionic surfactant and amino acid. The lyophilized formulations were evaluated for appearance, appearance after dissolution, and SE-HPLC analysis.

[262]

[263] For the preparation of the lyophilized formulations, each solution for formulations having the compositions shown in Table 2 below was aliquoted into a glass vial (20 mL) with 4.0 mL, half-capped with a rubber stopper, and then loaded on a shelf of a freeze dryer (Lyostar 3, SP scientific). Subsequently, lyophilization was conducted under the conditions shown in Table 3 below, and the prepared lyophilized formulation was capped with an aluminum cap after the completion of aluminum lyophilization.

[264]

[265] [Table 2]

#	Fusion protein (Fc-GLA (mg/mL))	Buffer (L-Histidine (mM))	Amino acid (L-Serine (%,(w/v)))	Non-ionic surfactant (Polysorbate 20 (%,(w/v)))	pH
1	30	10	2.1	0.001	6.0
2	30	10	2.1	0.001	7.0
3	30	10	2.1.	0.05	6.0
4	30	10	2.1	0.05	7.0
5-7	30	10	2.5	0.0255	6.5
8	30	10	2.9	0.001	6.0
9	30	10	2.9	0.001	7.0
10	30	10	2.9	0.05	6.0
11	30	10	2.9	0.05	7.0

[266]

[267] [Table 3]

	Loading	Freezing	Primary drying	Secondary drying
Temperature (°C)	5	-55	-25	25
Temperature change rate (°C/min)	0	0.33	0.33	0.25
Holding time (min)	30	300	4500	1200
Pressure (mTorr)	N/A	N/A	60	50

[268]

[269] Example 2-2: Dissolution and appearance of lyophilized formulations

[270]

[271] Each lyophilized vial was allowed to stand at room temperature for 30 minutes to remove the moisture generated on the vial surface, and then the appearance of a cake was examined.

[272] A 25G needle (1 inch or more) was fitted onto a 2 mL syringe, and 2 mL of distilled water was drawn out, and then injected into each vial containing the lyophilized product at a 90-degree angle. Since the vacuum was maintained inside the vial, the distilled water was automatically injected.

[273] Upon the completion of distilled water injection, the needle and syringe were separated to release the remaining vacuum inside the vial, and then the needle was removed. The vial was shaken 5 times and left to stand until the lyophilized cake dissolved.

[274] The appearance of the dissolved solution in the vial was examined, and then the vial was allowed to stand under refrigeration for 3 days, followed by subsequent appearance examination.

[275]

[276] Example 2-3: Size exclusion liquid chromatography (SE-HPLC)

[277]

[278] The lyophilized product dissolved in Example 2-2 was diluted using a mobile phase (1xPBS, Lonza) to a concentration of 1 mg/mL and then filtered using a 0.2-μm syringe filter. Thereafter, 200 μL of the filtered sample was injected into a vial insert and then prepared in a screw top vial.

[279] Subsequently, the mobile phase was connected to a pump, and then an assay column (TSKgel G3000SWXL, Tosoh) was mounted on Waters e2695 and Waters 2489 instruments (Waters) while the mobile phase flowed at a flow rate of 0.5 mL/min.

The sample was equilibrated by flowing the mobile phase at a flow rate of 0.5 mL/min for 30 minutes or longer until detector signals were stabilized. When the temperature of an autosampler was lowered to 4°C, the sample was placed into the sampler, and 10 µL of the sample was injected. Thereafter, the mobile phase was allowed to flow for 35 minutes, and detection peaks were identified at 214 nm. Analysis was performed by Empower Pro software on a PC.

[280]

[281] Example 2-4: Comparison of appearances and recovery rates of native form of lyophilized formulations according to pH changes

[282]

[283] First, as a result of comparing the cake appearance among the lyophilized formulations prepared in Example 2-2, the appearance was appropriate without serious collapse under all the conditions (FIG. 1).

[284] The appearance immediately after dissolution was transparent under all the conditions, but the appearance after 3 days of standing under refrigeration became more turbid as the pH decreased (FIG. 1). Specifically, the lyophilized formulations with a pH of 6.5 or higher showed a transparent appearance after dissolution.

[285]

[286] As a result of examining the recovery rate of the native form in the prepared lyophilized formulations by size exclusion chromatography, the recovery rate of the native form decreased as the pH was lowered (FIG. 2). Specifically, the lyophilized formulations with a pH of 6.5 or higher showed a high recovery rate of the native form. The native form indicates the α -galactosidase A fusion protein prepared in Example 1-1.

[287]

[288] The results of comparing the appearances and recovery rates of the native form of the lyophilized formulations according to pH changes are summarized in Table 4 below.

[289]

[290] [Table 4]

#	Cake appearance of lyophilized product	Appearance immediately after dissolution	Appearance after dissolution followed by storage for three days	Recovery rate of native form (%)
1	Elegant	Transparent	Very turbid	96.5
2	Elegant	Transparent	Transparent	97.8
3	Elegant	Transparent	Turbid	95.3
4	Elegant	Transparent	Transparent	98.0
5-7	Elegant	Transparent	Transparent	96.1
8	Elegant	Transparent	Very turbid	92.4
9	Elegant	Transparent	Transparent	95.7
10	Elegant	Transparent	Slightly turbid	87.8
11	Elegant	Transparent	Transparent	90.5

[291]

[292] **Example 3: Conditions for improving recovery rate of native form through pH, change in filling volume, and sugar screening**

[293]

[294] Example 3-1: Preparation of lyophilized formulations

[295]

[296] After it was confirmed that the appearance after dissolution was improved for the formulations with a pH of 6.5 or higher in the above Example 2, for the purpose of increasing the recovery rate of the native form, various lyophilized formulations were prepared by adding a buffer, an amino acid, a nonionic surfactant, varying filling volume, and three types of sugars (sucrose, trehalose, mannitol) to the final raw solution, and then size exclusion chromatography analysis was performed.

[297]

[298] Each solution for formulations having the compositions shown in Table 5 below was aliquoted into a glass vial (20 mL) with 4 mL or 6 mL, half-capped with a rubber stopper, and then loaded on a shelf of a freeze dryer (Lyostar 3, SP scientific). Subsequently, lyophilization was conducted under the conditions shown in Table 6 below, and the prepared lyophilized formulation was capped with an aluminum cap after the completion of aluminum lyophilization.

[299]

[300] [Table 5]

#	pH	Buffer (L-Histidine (mM))	Fusion protein (Fc-GLA (mg/mL))	Sugar	Filling amount (mL)	Amino acid (L-Serine (%,(w/v)))	Non-ionic surfactant (Polysorb ate 20 (%,(w/v)))
1	6.5	6.67	20	Sucrose 10 mg/mL	6	1.4	0.017
2		6.67		Trehalose 10 mg/mL			
3		6.67		Mannitol 10 mg/mL			
4		6.67		-			
5	6.8	6.67	20	Sucrose 10 mg/mL	6	1.4	0.017
6		6.67		Trehalose 10 mg/mL			
7		6.67		Mannitol 10 mg/mL			
8				-			
10	6.5	10	30	Sucrose 15 mg/mL	4	2.1	0.025
12		10		Trehalose 15 mg/mL			
14		10		Mannitol 15 mg/mL			
16		10		-			
18	6.8	10	30	Sucrose 15 mg/mL	4	2.1	0.025
20		10		Trehalose 15 mg/mL			
22		10		Mannitol 15 mg/mL			
24		10		-			

[301] [Table 6]

	Loading	Freezing	Primary drying	Secondary drying
Temperature (°C)	5	-55	-25	25
Temperature change rate (°C/min)	0	0.33	0.33	0.25
Holding time (min)	30	300	4500	1200
Pressure (mTorr)	N/A	N/A	60	50

[302]

[303] Example 3-2: Dissolution and appearance of lyophilized formulations

[304]

[305] Each lyophilized vial prepared in Example 3-1 was allowed to stand at room temperature for 30 minutes to remove the moisture generated on the vial surface, and then the appearance of a cake was examined.

[306] A 25G needle (1 inch or more) was fitted onto a 2 mL syringe, and 2 mL of distilled water was drawn out, and then injected into each vial containing the lyophilized product at a 90-degree angle. Since the vacuum was maintained inside the vial, the distilled water was automatically injected.

[307] Upon the completion of distilled water injection, the needle and syringe were separated to release the remaining vacuum inside the vial, and then the needle was removed. The vial was shaken 5 times and left to stand until the lyophilized cake dissolved.

[308]

[309] Example 3-3: Size exclusion liquid chromatography (SE-HPLC)

[310]

[311] The lyophilized product dissolved in Example 3-2 was diluted using a mobile phase (1xPBS, Lonza) to a concentration of 1 mg/mL and then filtered using a 0.2-μm syringe filter. Thereafter, 200 μL of the filtered sample was injected into a vial insert and then prepared in a screw top vial.

[312] Subsequently, the mobile phase was connected to a pump, and then an assay column (TSKgel G3000SWXL, Tosoh) was mounted on Waters e2695 and Waters 2489 instruments (Waters) while the mobile phase flowed at a flow rate of 0.5 mL/min. The sample was equilibrated by flowing the mobile phase at a flow rate of 0.5 mL/min for 30 minutes or longer until detector signals were stabilized. When the temperature of an autosampler was lowered to 4°C, the sample was placed into the sampler, and 10

μL of the sample was injected. Thereafter, the mobile phase was allowed to flow for 35 minutes, and detection peaks were identified at 214 nm. Analysis was performed by Empower Pro software on a PC.

[313]

[314] Example 3-4: Conditions for improving recovery rate of native form according to pH, changes in filling volume, and sugar type

[315]

[316] First, as a result of comparing the cake appearance among the lyophilized formulations prepared in Example 3-2, no serious collapse was observed under all the conditions (FIGS. 3 and 4).

[317]

[318] As a result of examining the recovery rate of the native form in the prepared lyophilized formulations by size exclusion chromatography, the proportion of the native form to the final raw solution was hardly decreased regardless of pH under the conditions containing three types of sugars, but the proportion of the native form to the final raw solution decreased under all the conditions not containing sugars (FIGS. 5 and 6).

[319] These results suggest that the lyophilized formulations containing the fusion protein of the present invention, which contain sugars, showed an excellent recovery rate of the native form regardless of the specific type of sugars.

[320]

[321] Additionally, as for the comparison of filling volume of the final raw material, the recovery rate of the native form was higher for a filling volume of 6 mL (FIGS. 5 and 6).

[322]

[323] **Example 4: Optimum concentration of sucrose (white sugar) for improving recovery rate of native form**

[324]

[325] Example 4-1: Preparation of lyophilized formulations

[326]

[327] After it was confirmed that the inclusion of a sugar at a pH of 6.8 showed an excellent recovery rate of the native form, various lyophilized formulations were prepared by adjusting the amount of sucrose (white sugar) as a representative example of saccharides, and then size exclusion chromatography analysis was performed.

[328]

[329] Each solution for formulations having the compositions shown in Table 7 below was aliquoted into a glass vial (20 mL) with 6 mL, half-capped with a rubber stopper, and then loaded on a shelf of a freeze dryer (Lyostar 3, SP scientific). Subsequently,

lyophilization was conducted under the conditions shown in Table 7 below, and the prepared lyophilized formulation was capped with an aluminum cap after the completion of aluminum lyophilization.

[330]

[331] [Table 7]

#	pH	Buffer (L-Histidine (mM))	Fusion protein (Fc-GLA (mg/mL))	White sugar (mg/mL)	Total Filling amount (mL)	Amino acid (L-Serine (%,(w/v)))	Non-ionic surfactant (Polysorbate 20 (%,(w/v)))
1	6.8	6.67	20	0	6	1.4	0.017
2		6.67		2.5			
3		6.67		5			
4		6.67		10			

[332]

[333] [Table 8]

	Loading	Freezing	Primary drying	Secondary drying
Temperature (°C)	5	-55	-25	25
Temperature change rat (°C/min)	0	0.33	0.33	0.25
Holding time (min)	30	300	2500	1200
Pressure (mTorr)	N/A	N/A	60	50

[334]

[335] Example 4-2: Dissolution and appearance of lyophilized formulations

[336]

[337] Each lyophilized vial was allowed to stand at room temperature for 30 minutes to remove the moisture generated on the vial surface, and then the appearance of a cake was examined.

[338] A 25G needle (1 inch or more) was fitted onto a 2 mL syringe, and 2 mL of distilled water was drawn out, and then injected into each vial containing the lyophilized product at a 90-degree angle. Since the vacuum was maintained inside the vial, the distilled water was automatically injected.

- [339] Upon the completion of distilled water injection, the needle and syringe were separated to release the remaining vacuum inside the vial, and then the needle was removed. The vial was shaken 5 times and left to stand until the lyophilized cake dissolved.
- [340]
- [341] Example 4-3: Size exclusion liquid chromatography (SE-HPLC)
- [342]
- [343] The lyophilized product dissolved in Example 4-2 was diluted using a mobile phase (1xPBS, Lonza) to a concentration of 1 mg/mL and then filtered using a 0.2- μ m syringe filter. Thereafter, 200 μ L of the filtered sample was injected into a vial insert and then prepared in a screw top vial.
- [344] Subsequently, the mobile phase was connected to a pump, and then an assay column (TSKgel G3000SWXL, Tosoh) was mounted on Waters e2695 and Waters 2489 instruments (Waters) while the mobile phase flowed at a flow rate of 0.5 mL/min. The sample was equilibrated by flowing the mobile phase at a flow rate of 0.5 mL/min for 30 minutes or longer until detector signals were stabilized. When the temperature of an autosampler was lowered to 4°C, the sample was placed into the sampler, and 10 μ L of the sample was injected. Thereafter, the mobile phase was allowed to flow for 35 minutes, and detection peaks were identified at 214 nm. Analysis was performed by Empower Pro software on a PC.
- [345]
- [346] Example 4-4: Optimum concentration of white sugar for improving recovery rate of native form
- [347]
- [348] First, as a result of comparing the cake appearance among the lyophilized formulations prepared in Example 4-2, no serious collapse was observed under all the conditions (FIG. 7).
- [349] As a result of examining the recovery rate of the native form in the prepared lyophilized formulations by size exclusion chromatography, the proportion of the native form to the final raw solution was least reduced under the condition that contains white sugar at a concentration of 2.5 mg/mL (#2) (FIG. 8).
- [350]
- [351] **Example 5: Long-term storage stability of optimum lyophilized formulation**
- [352]
- [353] Example 5-1: Preparation of lyophilized formulation
- [354]
- [355] Considering the optimum recovery conditions of the native form derived through the previous examples, an optimum lyophilized formulation was prepared, and then

subjected to size exclusion chromatography analysis at regular intervals while stored under refrigeration conditions ($5\pm 3^{\circ}\text{C}$).

[356]

[357] Specifically, a solution for a formulation having the composition shown in Table 9 below was aliquoted into a glass vial (20 mL) with 6 mL, half-capped with a rubber stopper, and then loaded on a shelf of a freeze dryer (Lyostar 3, SP scientific). Subsequently, lyophilization was conducted under the conditions shown in Table 10 below, and the prepared lyophilized formulation was capped with an aluminum cap after the completion of aluminum lyophilization.

[358]

[359] [Table 9]

#	pH	Buffer (L-Histidine (mM))	Fusion protein (Fc-GLA (mg/mL))	White sugar (mg/mL)	Amino acid (L-Serine (%,(w/v)))	Non-ionic surfactant (Polysorbate 20 (%,(w/v)))
1	6.8	6.67	22	2.5	1.4	0.017

[360]

[361] [Table 10]

	Loading	Freezing	Primary drying	Secondary drying
Temperature ($^{\circ}\text{C}$)	5	-55	-25	25
Temperature change rate ($^{\circ}\text{C}/\text{min}$)	0	0.33	0.33	0.25
Holding time (min)	30	300	2500	1200
Pressure (mTorr)	N/A	N/A	60	50

[362]

[363] Example 5-2: Dissolution and appearance of lyophilized formulations

[364]

[365] The lyophilized vial was allowed to stand at room temperature for 30 minutes to remove the moisture generated on the vial surface, and then the appearance of a cake was examined.

[366] A 25G needle (1 inch or more) was fitted onto a 2 mL syringe, and 2 mL of distilled water was drawn out, and then injected into each vial containing the lyophilized

product at a 90-degree angle. Since the vacuum was maintained inside the vial, the distilled water was automatically injected.

[367] Upon the completion of distilled water injection, the needle and syringe were separated to release the remaining vacuum inside the vial, and then the needle was removed. The vial was shaken 5 times and left to stand until the lyophilized cake dissolved.

[368]

[369] Example 5-3: Moisture content

[370]

[371] Three lyophilized vials for each condition were placed in a moisture content analysis room controlled to maintain a humidity of 40% or less, for at least one hour. Three sets of 885 KF Thermoprep vials and caps to be used as a control group (Blank) were prepared.

[372] The lyophilized cake in each of the vials was crushed as finely as possible using a spatula, transferred to a zero-calibrated 885 KF Thermoprep vial, weighed, and capped. The control vials were capped without separate weighing. Thereafter, the prepared vials were placed in the auto-sampler in the order of the control group followed by the sample group.

[373] The 831 KF Coulometer Remote Controller was programmed to measure each control vial three times and each test sample three times. The total number of samples to be analyzed was entered into the 885 Compact Oven, and then analysis was performed.

[374]

[375] Example 5-4: Size exclusion liquid chromatography (SE-HPLC)

[376]

[377] The lyophilized product dissolved in Example 5-2 was diluted using a mobile phase (1xPBS, Lonza) to a concentration of 1 mg/mL and then filtered using a 0.2- μ m syringe filter. Thereafter, 200 μ L of the filtered sample was injected into a vial insert and then prepared in a screw top vial.

[378] Subsequently, the mobile phase was connected to a pump, and then an assay column (TSKgel G3000SWXL, Tosoh) was mounted on Waters e2695 and Waters 2489 instruments (Waters) while the mobile phase flowed at a flow rate of 0.5 mL/min. The sample was equilibrated by flowing the mobile phase at a flow rate of 0.5 mL/min for 30 minutes or longer until detector signals were stabilized. When the temperature of an autosampler was lowered to 4°C, the sample was placed into the sampler, and 10 μ L of the sample was injected. Thereafter, the mobile phase was allowed to flow for 35 minutes, and detection peaks were identified at 214 nm. Analysis was performed by Empower Pro software on a PC.

[379]

[380] Example 5-5: Long-term storage stability of optimum lyophilized formulation

[381]

[382] As a result of analyzing the appearance and moisture content of the lyophilized formulation prepared in Example 5-1, the appearance of the cake or the appearance after dissolution was maintained as transparent even after 12 months under refrigeration ($5\pm 3^{\circ}\text{C}$) conditions, and the moisture content was also maintained at 1.0% even after 6 months (FIG. 9).

[383] The prepared lyophilized formulation was stored under refrigeration ($5\pm 3^{\circ}\text{C}$), and after 3, 6, and 12 months, the recovery rate of the native form was examined by size exclusion chromatography. As a result, the proportion of the native form was maintained with little reduction even after 12 months compared with 0 months (FIG. 10).

[384]

[385] While the present invention has been described with reference to the particular illustrative embodiments, a person skilled in the art to which the present invention pertains can understand that the present invention may be embodied in other specific forms without departing from the technical spirit or essential characteristics thereof. Therefore, the exemplary embodiments described above should be construed as being exemplified and not limiting the present disclosure. The scope of the invention should be construed that the meaning and scope of the appended claims rather than the detailed description and all changes or variations derived from the equivalent concepts fall within the scope of the present invention.

Claims

- [Claim 1] A lyophilized formulation comprising a fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region, the lyophilized formulation comprising a mixture obtained by lyophilizing an aqueous solution containing:
the fusion protein at a concentration of 10 to 40 mg/mL;
a buffer with a pH of 6.5 to 8.0; and
a sugar or sugar alcohol at a concentration of 1 to 20 mg/mL.
- [Claim 2] The lyophilized formulation of claim 1, wherein the sugar is glucose, fructose, galactose, lactose, maltose, sucrose, trehalose, or a combination thereof.
- [Claim 3] The lyophilized formulation of claim 1, wherein the sugar alcohol is mannitol, sorbitol, or a combination thereof.
- [Claim 4] The lyophilized formulation of claim 1, wherein the aqueous solution further contains an amino acid at a concentration of 1.0 to 3.0% (w/v).
- [Claim 5] The lyophilized formulation of claim 4, wherein the amino acid is selected from the group consisting of serine, arginine, threonine, glutamine, glycine, alanine, and a combination thereof.
- [Claim 6] The lyophilized formulation of claim 1, wherein the aqueous solution further contains a non-ionic surfactant at a concentration of 0.001 to 0.1% (w/v).
- [Claim 7] The lyophilized formulation of claim 6, wherein the non-ionic surfactant is selected from the group consisting of poloxamer 188, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, and a combination thereof.
- [Claim 8] The lyophilized formulation of claim 1, wherein the buffer contains histidine or a salt thereof, citric acid or a salt thereof, acetic acid or a salt thereof, phosphoric acid or a salt thereof, or a combination thereof.
- [Claim 9] The lyophilized formulation of claim 1, wherein the buffer contains 5 to 30 mM histidine.
- [Claim 10] The lyophilized formulation of claim 1, wherein the lyophilized formulation comprises a mixture obtained by lyophilizing 1 to 10 mL of an aqueous solution.
- [Claim 11] The lyophilized formulation of claim 1, wherein the aqueous solution does not further contain an isotonic agent.

- [Claim 12] The lyophilized formulation of claim 1, wherein the α -galactosidase A includes the amino acid sequence of SEQ ID NO: 1.
- [Claim 13] The lyophilized formulation of claim 1, wherein the fusion protein includes the amino acid sequence of SEQ ID NO: 4.
- [Claim 14] The lyophilized formulation of claim 1, wherein the fusion protein has a structure in which two molecules of α -galactosidase A are linked to monomers of an immunoglobulin Fc region in a dimeric form, respectively.
- [Claim 15] The lyophilized formulation of claim 1, wherein the lyophilized formulation comprises a mixture obtained by lyophilizing an aqueous solution containing:
the fusion protein at a concentration of 15 to 35 mg/mL;
histidine at a concentration of 5 to 30 mM;
the sugar or sugar alcohol at a concentration of 2.5 to 10 mg/mL;
polysorbate 20 at a concentration of 0.001 to 0.05% (w/v); and
serine at a concentration of 1.0 to 3.0% (w/v).
- [Claim 16] The lyophilized formulation of claim 1, wherein the lyophilized formulation is used to prevent or treat Fabry disease.
- [Claim 17] A method for preparing the lyophilized formulation of any one of claims 1 to 16, the method comprising lyophilizing an aqueous solution containing i) an α -galactosidase A fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region, ii) a buffer, and iii) a sugar or sugar alcohol.
- [Claim 18] The method of claim 17, wherein the method comprises lyophilizing 1 to 10 mL of an aqueous solution containing i) an α -galactosidase A fusion protein in which α -galactosidase A is linked to an immunoglobulin Fc region, ii) a buffer, and iii) a sugar or sugar alcohol.
- [Claim 19] A method for reconstituting the lyophilized formulation of any one of claims 1 to 18, the method comprising adding a reconstitution solution to the lyophilized formulation of any one of claims 1 to 18.
- [Claim 20] The method of claim 19, wherein the reconstitution solution is distilled water.
- [Claim 21] The method of claim 19, wherein the formulation reconstituted by the method contains the α -galactosidase A fusion protein at a concentration of 10 to 100 mg/mL.