

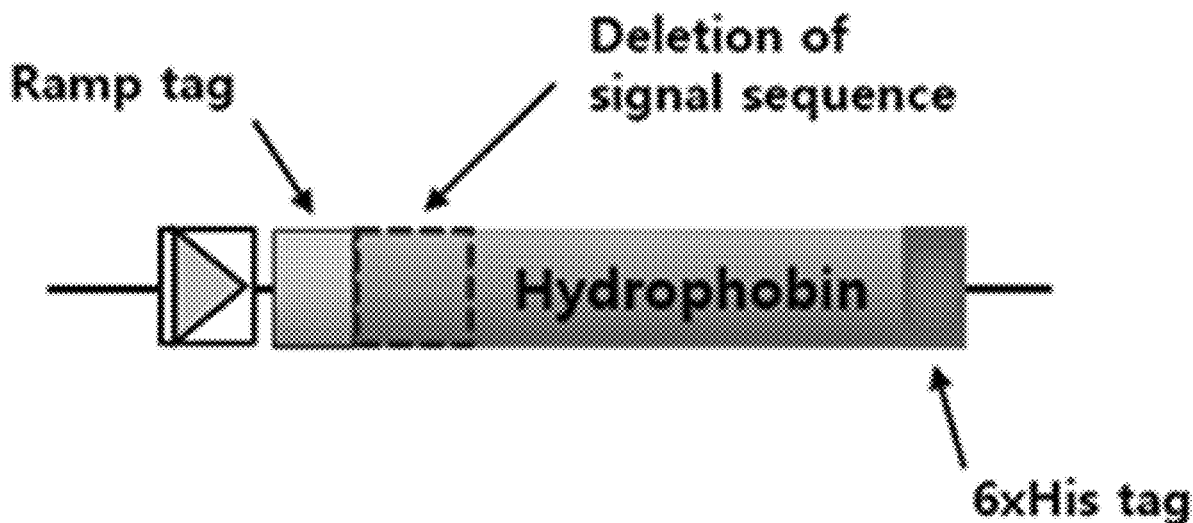


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(19) **United States**(12) **Patent Application Publication** (10) **Pub. No.: US 2021/0363199 A1**
(43) **Pub. Date: Nov. 25, 2021**(54) **METHOD FOR SOLUBLE EXPRESSION AND PURIFICATION OF HYDROPHOBIN****Publication Classification**(71) Applicant: **INDUSTRY FOUNDATION OF CHONNAM NATIONAL UNIVERSITY**, Gwangju (KR)(51) **Int. Cl.**
C07K 14/37 (2006.01)
C07K 7/06 (2006.01)
(52) **U.S. Cl.**
CPC *C07K 14/37* (2013.01); *C07K 2319/00* (2013.01); *C07K 7/06* (2013.01)(72) Inventors: **Geun Joong KIM**, Gwangju (KR); **Dae Eun CHEONG**, Gwangju (KR);
Ho-Dong LIM, Jeongeup-si (KR);
Sang-Oh AHN, Gwangju (KR)(57) **ABSTRACT**(21) Appl. No.: **16/944,078**(22) Filed: **Jul. 30, 2020**(30) **Foreign Application Priority Data**

May 20, 2020 (KR) 10-2020-0060264

A method of expressing hydrophobin in a soluble form and a method of purifying hydrophobin, and more particularly, to a method of expressing a recombinant fusion protein including hydrophobin in a soluble form in a host cell and then purifying the expressed recombinant fusion protein are provided.

Specification includes a Sequence Listing.

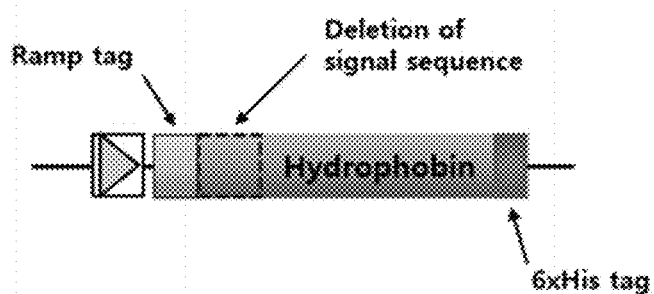


FIG. 1

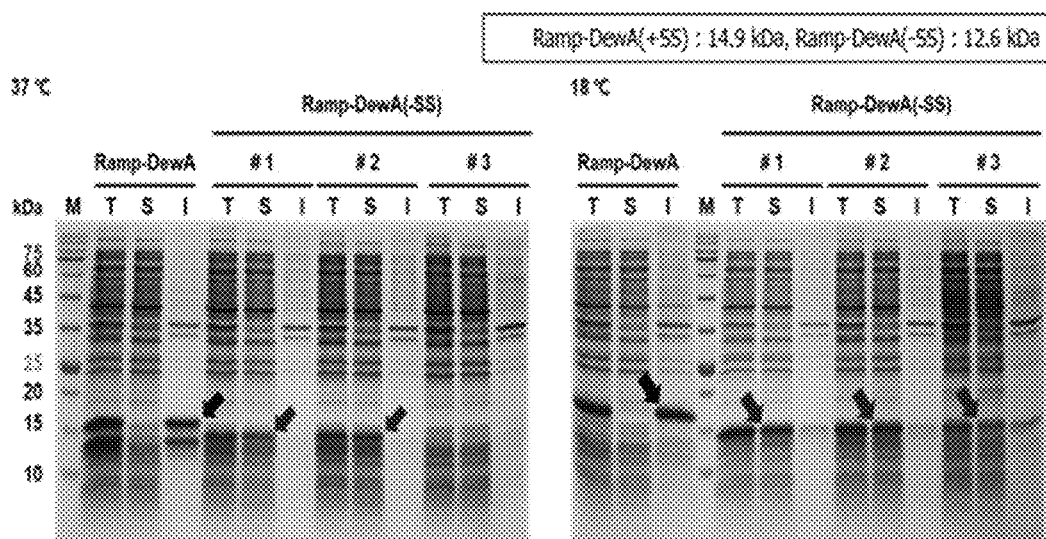


FIG. 2A

FIG. 2B

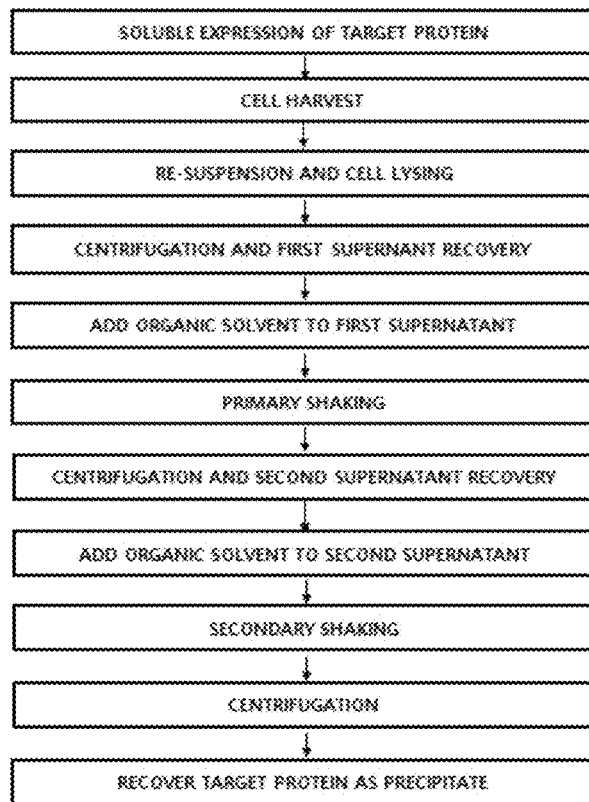


FIG. 3

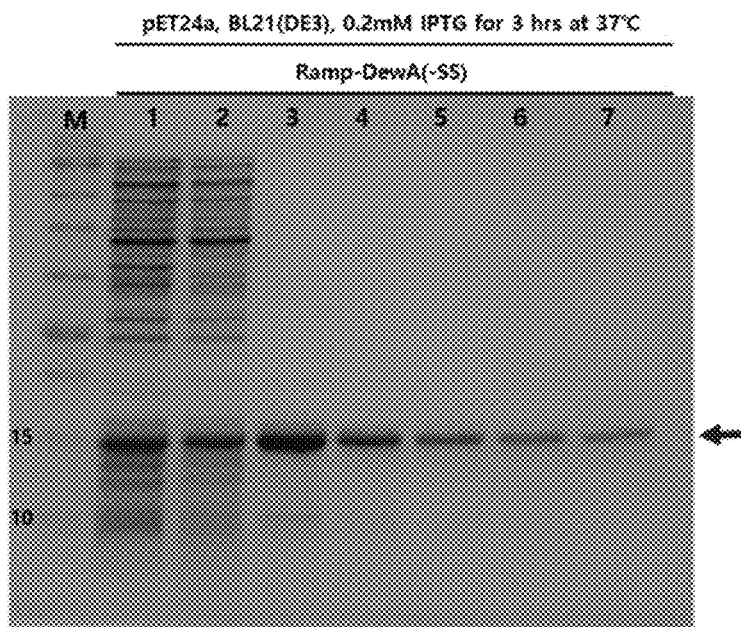


FIG. 4

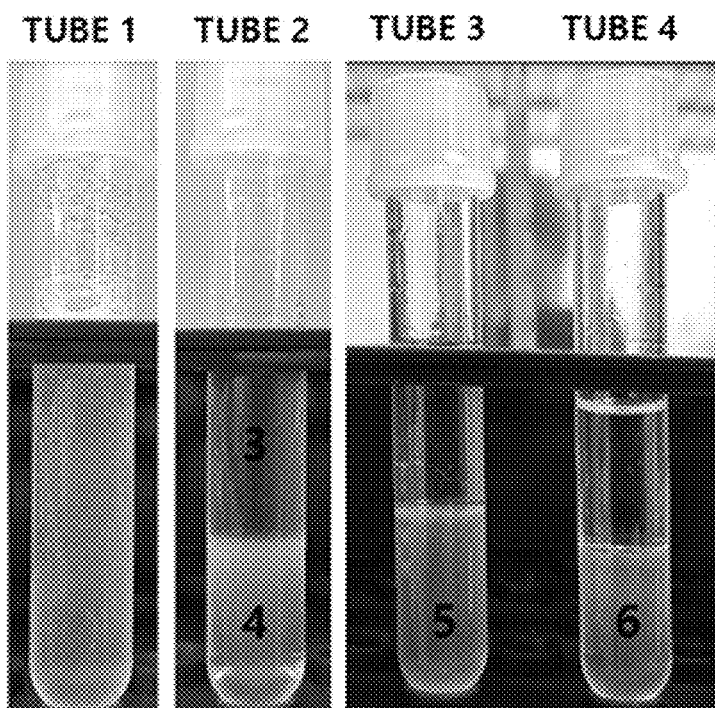


FIG. 5

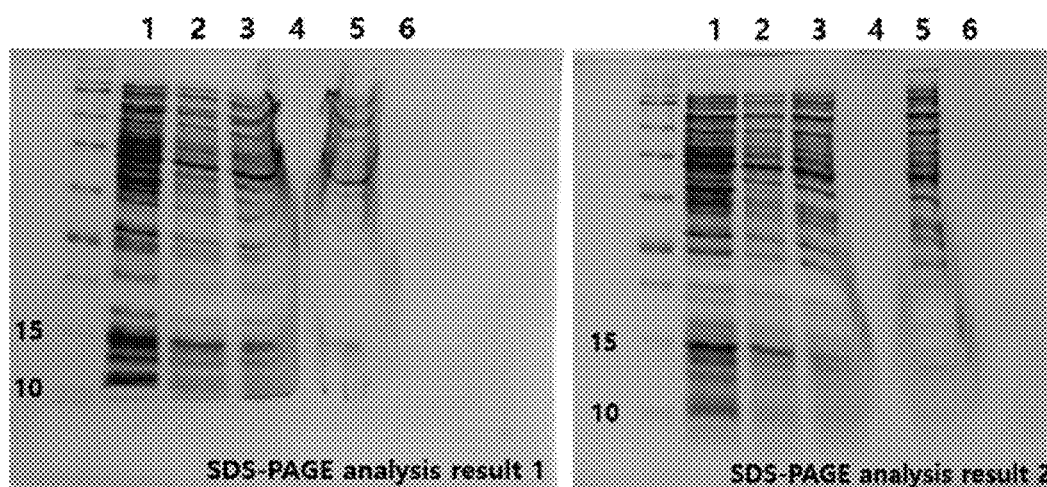


FIG. 6A

FIG. 6B

METHOD FOR SOLUBLE EXPRESSION AND PURIFICATION OF HYDROPHOBIN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119 to Korean Patent Application No. 10-2020-0060264, filed on May 20, 2020, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002] The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named Sequence_Listing_PLS20312.txt created on Jul. 30, 2020, and having a size of 6423 bytes and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[0003] The following disclosure relates to a method of expressing hydrophobin in a soluble form and a method of purifying hydrophobin, and more particularly, to a method of expressing a recombinant fusion protein including hydrophobin in a soluble form in a host cell and then purifying the expressed recombinant fusion protein.

BACKGROUND

[0004] Hydrophobin is a small protein initially isolated from *Shizophyllum commune*. The hydrophobin is composed of 100 to 150 amino acids, and is mainly expressed in a mycelia fungus. In hyphae growth of fungi, the hydrophobin which is amphipathic forms a hydrophobic surface layer through self-assembly when a monomer thereof is secreted to the outside. As a result, the hydrophobin acts to coat a surface of the hypha through an amphiphilic polymer composition. Through the above action, the hydrophobin not only enables the hypha to effectively respond to changes in the surrounding environment but also functions as a shield between a cell wall and an air layer or at an interface between a cell wall and a solid surface during sporulation, fruiting body development, and host invasion through infection structure formation (Bayry et al., 2012).

[0005] The hydrophobin forms four disulfide bonds in a molecule from eight cysteine residues included in a protein molecule. These disulfide bonds play an important role in stabilization of an amphipathic three-dimensional structure that imparts activity similar to a surfactant in the hydrophobin, which enables the hydrophobin to be self-assembled into an amphipathic monolayer at a hydrophilic-hydrophobic interface. Hydrophobin is classified into Class I and Class II depending on a hydrophobicity plot, solubility, and a structure formed during self-assembly. Both Class I and Class II hydrophobins form an amphipathic monolayer at a hydrophilic-hydrophobic interface; however, Class I hydrophobin forms amyloid-like rodlets which are insoluble at a hydrophilic-hydrophobic interface, whereas, Class II hydrophobin forms a monolayer having a high solubility at a hydrophilic-hydrophobic interface.

[0006] Due to the above features, hydrophobin has been spotlighted in the biomaterial industry. Accordingly, the use

of hydrophobin in cosmetics or food and beverage requiring stable and uniform foam has been actively studied. In addition, hydrophobin has received increasing attention as a next-generation innovative material such as a medical coating agent or a functional particle for a nano-structure applied to a living body in various industries.

[0007] Until now, methods of producing hydrophobin using a bacterium, a plant cell, yeast, or the like have been attempted, in addition to a method using mold which is the original strain as it is. In particular, a protein to be produced in a bacterium in an inclusion body form has been purified for use. However, current methods require a denaturation/refolding process to obtain a final hydrophobin protein, which means a significant increase in costs in the mass production of hydrophobin.

[0008] For example, it has been reported by BASF SE in 2009 that two recombinant Class I hydrophobins such as H*Protein A (yaaD—*A. nidulans* DewA-His₆) and H*Protein B (cleaved yaaD—*A. nidulans* DewA-His₆) are able to be produced to the extent of industrial applicability using a bacterium as a host; however, in this case, hydrophobin is also produced in an inclusion body form. Until now, there have been no reports of a case in which a recombinant hydrophobin is successfully expressed in a soluble form (Wendel et al. 2010).

[0009] In addition to the problem above, HPLC, two phase separation method (ATPS; aqueous two-phase systems) using a surfactant have been mainly used for purifying hydrophobin in the related art, but it takes a lot of time and cost during the purification due to a complex procedure and use of chemical materials that are not easy to handle. Therefore, these problems are required to be urgently solved.

RELATED ART DOCUMENT

Patent Document

[0010] (Patent Document 1) Korean Patent Publication No. 1446054 (2014 Sep. 24)

[0011] (Patent Document 2) Korean Patent Laid-Open Publication No. 2014-0022839

Non-Patent Document

[0012] (Non-Patent Document 1) Bayry J. et al. PLOS Pathog 8(5):e1002700 (2012)

[0013] (Non-Patent Document 2) Linder M. B. et al. FEMS Microbiol Rev 29(5):877-896 (2005)

[0014] (Non-Patent Document 3) Kwan A H Y et al. PNAS USA 2006; 103:3621 LP 3626 (2006)

[0015] (Non-Patent Document 4) Wendel W. et al. Eur. Biophys. J. 39:457-468 (2010)

SUMMARY

[0016] An embodiment of the present invention is directed to providing a recombinant fusion protein for soluble expression of hydrophobin in a heterologous host, a method of producing the same, and a method of purifying soluble hydrophobin through the production method.

[0017] In one general aspect, a recombinant fusion protein expressed in a soluble form includes: a target protein; and a ramp tag for controlling a translation speed, fused at an N-terminal of the target protein, wherein a signal sequence

of the N-terminal of the target protein is subjected to mutation including the deletion.

[0018] The target protein may be hydrophobin.

[0019] The target protein may be Class I hydrophobin.

[0020] The target protein may be DewA.

[0021] A wild type target protein may consist of an amino acid sequence of SEQ ID NO: 2.

[0022] The target protein may consist of an amino acid sequence of SEQ ID NO: 8 or 10.

[0023] The ramp tag may consist of an amino acid sequence of SEQ ID NO: 5.

[0024] The signal sequence may consist of a base sequence of SEQ ID NO: 3.

[0025] The mutation may be one or two or more selected from the group consisting of a substitution and a deletion of a part or all of amino acids of the signal sequence and an addition of a new amino acid.

[0026] In another general aspect, there is provided a base sequence encoding the recombinant fusion protein.

[0027] In still another general aspect, there is provided a vector for soluble expression of the recombinant fusion protein, the base sequence being introduced into the vector.

[0028] In still another general aspect, there is provided a transformant for soluble expression of the recombinant fusion protein, the transformant being transformed with the vector.

[0029] In still another general aspect, there is provided a method of purifying a recombinant fusion protein expressed in a soluble form, the method including: a) expressing a recombinant fusion protein in a soluble form by transforming a non-human host cell with a vector into which a base sequence encoding a recombinant fusion protein is introduced, the recombinant fusion protein including: a target protein; and a ramp tag for controlling a translation rate, fused at an N-terminal of the target protein; and b) purifying the expressed recombinant fusion protein.

[0030] Step b) above may include: b1) suspending the transformed cell and lysing and centrifuging the suspended cell to obtain a first supernatant; b2) shaking and then centrifuging a first mixture obtained by adding a first solvent to the first supernatant to obtain a second supernatant; and b3) shaking and then centrifuging a second mixture obtained by adding a second solvent to the second supernatant to recover the target protein.

[0031] The target protein may be hydrophobin.

[0032] The target protein may be Class I hydrophobin.

[0033] The target protein may be DewA.

[0034] A wild type target protein may consist of an amino acid sequence of SEQ ID NO: 2.

[0035] The target protein may consist of an amino acid sequence of SEQ ID NO: 8 or 10.

[0036] The ramp tag may be obtained by collecting a rare codon of the host cell.

[0037] The host cell may be a bacterium belonging to *Escherichia* sp., *Salmonellae* sp., *Yersinia* sp., *Shigella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Proteus* sp., or *Klebsiella* sp.

[0038] Each of the first solvent and the second solvent may be selected from the group consisting of C3 organic solvents.

[0039] A volume ratio of the organic solvent to the first supernatant may be 1:2 or 1:3.

[0040] The ramp tag may consist of an amino acid sequence of SEQ ID NO: 5.

[0041] The rare codon may be collected by analyzing a frequency of the codon and the number of isoacceptor tRNA genes.

[0042] The frequency of the codon may be 0.1 to 1%. The number of isoacceptor tRNA genes may be 0 to 2.

[0043] The target protein may be a physiologically active protein including hormones and receptors thereof, biological response modifiers and receptors thereof, cytokines and receptors thereof, enzymes, antibodies, and antibody fragments.

[0044] In still another general aspect, there is provided a recombinant fusion protein expressed in a soluble form, the recombinant fusion protein being purified by the method.

[0045] Other features and aspects will be apparent from the following detailed description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] FIG. 1 is a schematic view of a structure of a recombinant fusion protein for soluble expression of hydrophobin.

[0047] FIGS. 2A and 2B show SDS-PAGE analysis results after culturing at (a) 37° C. and (b) 18° C. to confirm soluble expression of hydrophobin.

[0048] FIG. 3 illustrates an example of a flowchart of a process of purifying the soluble hydrophobin expressed according to the present invention.

[0049] FIG. 4 shows an SDS-PAGE analysis result for confirming purification efficiency of the soluble hydrophobin purified according to the present invention.

[0050] FIG. 5 illustrates a purification procedure of hydrophobin by a two phase separation method using Triton X-114 known in the related art.

[0051] FIGS. 6 A and B shows SDS-PAGE analysis results for confirming purification efficiency of the hydrophobin purified by the two phase separation method using the Triton X-114.

DETAILED DESCRIPTION

[0052] Hereinafter, a method of expressing hydrophobin in a soluble form and a method of purifying hydrophobin according to the present invention will be described in detail with reference to the accompanying drawings.

[0053] The drawings to be described below are provided by way of example so that the spirit of the present invention can be sufficiently transferred to those skilled in the art. Therefore, the present invention is not limited to the drawings suggested below but may be modified in many different forms. In addition, the drawings suggested below will be exaggerated in order to clarify the spirit of the present invention.

[0054] Technical terms and scientific terms used in the specification of the present invention have the general meanings understood by those skilled in the art to which the present invention pertains unless otherwise defined, and a description for the known function and configuration obscuring the present invention will be omitted in the following description and the accompanying drawings. In addition, singular forms used in the specification of the present invention may be intended to include plural forms unless otherwise specified. In addition, a unit used in the specification of the present invention unless otherwise speci-

fied is based on a weight. As an example, % or a unit of a ratio refers to wt % or a weight ratio.

[0055] The term “target protein” in the present invention is a protein intended to be produced in large quantities by those skilled in the art, and refers to any protein that can be expressed in a transformant by inserting polynucleotide encoding the protein to a recombinant expression vector.

[0056] In addition, the term “recombinant protein” or “fusion protein” refers to a protein to which another protein is connected to an N-terminal or a C-terminal of an initial target protein sequence, or refers to a protein to which another amino acid sequence is added. In addition, the above term refers to a recombinant fusion protein of the present invention obtained by linking a fusion partner to a target protein, or refers to a recombinant protein of a target protein from which a fusion partner is removed by a protein cleaving enzyme.

[0057] The term “vector” or “expression vector” is a linear or circular DNA molecule consisting of fragments encoding polypeptide, the polypeptide consisting of elements and additional fragments provided for gene transcription and translation and being linked to the DNA molecule to be operable. The additional fragment includes a promoter and a transcription termination signal sequence. The vector or the expression vector includes one or more replication origins, one or more selection markers, and the like. In general, the vector or the expression vector is derived from plasmid or virus DNA or from both of them.

[0058] The expression “comprise(s)” is intended to be open-ended transitional phrase having an equivalent meaning to an expression such as “include(s)”, “contain(s)”, “have(has)”, or “is (are) characterized”, and does not exclude elements, materials, or steps, which are not further recited. In addition, the expression “substantially consist(s) of” means that one specific element, material, and step, which are not recited with a specific element, material, and step, may be present at an amount having no unacceptably significant influence on at least one basic and novel technical idea of the invention. In addition, the expression “consist(s) of” means the presence of only the defined element, material, and step.

[0059] The terms “sample” and “specimen” in the present invention refer to subjects for analysis and are interchangeably used throughout the specification.

[0060] Hereinafter, a recombinant fusion protein expressed in a soluble form according to the present invention will be described in detail.

[0061] The recombinant fusion protein expressed in a soluble form is a recombinant fusion protein including a target protein and a ramp tag for controlling a translation rate, fused at an N-terminal of the target protein. A signal sequence of the N-terminal of the target protein is subjected to mutation.

[0062] In the recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the target protein may be hydrophobin, more preferably a Class I hydrophobin, and still more preferably a Class I DewA, and a wild type target protein may consist of an amino acid sequence of SEQ ID NO: 2. According to an exemplary embodiment of the present invention, the target protein of which the N-terminal has the mutated signal sequence may have an amino acid sequence of SEQ ID NO: 8 or 10, but the scope of the present invention is not limited thereto.

[0063] In the recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the ramp tag may be composed of 1 to 20 amino acids, preferably 6 to 12 amino acids, and more preferably 6 to 8 amino acids, but is not limited thereto. In the recombinant fusion protein according to an exemplary embodiment of the present invention, the ramp tag may consist of an amino acid sequence of SEQ ID NO: 5.

[0064] In the present invention, a ramp tag for controlling a translation rate for expressing a recombinant fusion protein, a method used for an application thereof, and the like is disclosed in Korean Patent Publication No. 1446054 registered by the present applicant, the contents of which are incorporated herein by reference in their entirety. In a case where the contents corresponding to the detailed description of the present invention are not particularly described, the method used in the above patent publication or a method appropriately modified to meet the object of the present invention, and the like may be applied.

[0065] As a specific example related to the content disclosed in Korean Patent Publication No. 1446054, a ramp tag for controlling a translation rate is prepared by using a method including: making a rare codon table according to a host cell; converting a DNA sequence of a target gene into codons; analyzing a frequency and position at which rare codons in the rare codon table appear in an open reading frame (ORF) of the target gene; and collecting and arranging the rare codons. Then, a protein that has a high added value but is difficult to be expressed, for example, esterase, β -glucosidase, cytolysin A, single chain Fv (scFv), asparaginase B, tetra-cell adhesion molecule (T-CAM), or B3(Fv)PE38, is effectively expressed by using the ramp tag.

[0066] In the present invention, the ramp tag may be prepared in a form in which the ramp tag for controlling a translation rate of the above patent publication is fused at the N-terminal of the target protein. By doing so, the ramp tag may be included in the recombinant fusion protein.

[0067] In the recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the signal sequence may be confirmed by a general method such as SignalP (see SignalP 5.0 improves signal peptide predictions using deep neural networks. José Juan Almagro Armenteros, Konstantinos D. Tsirigos, Casper Kaae Sønderby, Thomas Nordahl Petersen, Ole Winther, Søren Brunak, Gunnar von Heijne and Henrik Nielsen. *Nature Biotechnology*, 37, 420-423 (2019)) which is an exocrine signal sequence prediction program, but the present invention is not limited thereto. According to an exemplary embodiment of the present invention, the signal sequence may consist of a base sequence of SEQ ID NO: 3.

[0068] In the recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the mutation may be one or two or more selected from the group consisting of a substitution and a deletion of a part or all of amino acids of the signal sequence and an addition of a new amino acid.

[0069] According to an exemplary embodiment of the present invention, the mutation may be a deletion of a part or all of amino acids of the signal sequence, and for example, the 1st amino acid to the 18th amino acid of a sequence may be deleted. According to an exemplary embodiment of the present invention, as a specific example, the entire signal sequence corresponding to the underlined

part of SEQ ID NO: 1 shown in Table 1, below, may be deleted, but the present invention is not limited thereto.

[0070] According to an exemplary embodiment of the present invention, FIG. 1 illustrates an example of a structure of a recombinant fusion protein for soluble expression of a target protein. The ramp tag is fused at an N-terminal of the hydrophobin used as the target protein. The entire signal sequence of the hydrophobin is deleted. Various types of amino acid sequences that may be used for detecting and purifying a protein may be fused at a C-terminal of the hydrophobin used as the target protein, and for example, one or two or more tags or proteins selected from the group consisting of His tag, T7 tag, S-tag, Flag-tag, HA-tag, V5 epitope, PelB, and Xpress epitope may be used. Preferably, codons with respect to tags coded with six histidines (6×His tag) may be connected, but the present invention is not limited thereto.

[0071] From examples to be described below, it was newly confirmed that, in the case of the recombinant fusion protein expressed in a soluble form according to the present invention, a target protein expressed only in an insoluble inclusion body form in the related art is expressed in a soluble protein form. Accordingly, the present inventors solved the fundamental problem such as protein refolding which was the existing problem, and confirmed that a target protein having the original activity may be obtained in a high yield. Further, the present inventors have established a process capable of purifying a target protein with a significantly simplified procedure and high separation yield as compared with a process of purifying the target protein in the insoluble inclusion body form according to the related art.

[0072] The present invention provides a base sequence encoding the recombinant fusion protein. According to an exemplary embodiment of the present invention, for example, the base sequence may have a base sequence of SEQ ID NO: 1.

[0073] In addition, the present invention provides a vector for soluble expression of the protein, the base sequence being introduced into the vector.

[0074] In addition, the present invention provides a transformant for soluble expression of the recombinant fusion protein, the transformant being transformed with the vector. According to an exemplary embodiment of the present invention, the type of the transformant is not particularly limited as long as it may achieve the object of the present invention. However, an individual facilitated for a gene expression, for example, *Escherichia coli* or the like may be used.

[0075] Hereinafter, a method of purifying a recombinant fusion protein expressed in a soluble form according to the present invention will be described in detail.

[0076] The method of purifying a recombinant fusion protein expressed in a soluble form according to the present invention includes: a) expressing a recombinant fusion protein in a soluble form by transforming a non-human host cell with a vector into which a base sequence encoding a recombinant fusion protein is introduced, the recombinant fusion protein including: a target protein; and a ramp tag for controlling a translation rate, fused at an N-terminal of the target protein; and b) purifying the expressed recombinant fusion protein.

[0077] In the method of purifying a recombinant fusion protein expressed in a soluble form according to the present invention, step b) above may include: b1) suspending the

transformed cell and lysing and centrifuging the suspended cell to obtain a first supernatant; b2) shaking and then centrifuging a first mixture obtained by adding a first solvent to the first supernatant to obtain a second supernatant; and b3) shaking and then centrifuging a second mixture obtained by adding a second solvent to the second supernatant to recover the target protein.

[0078] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the target protein may consist of an amino acid sequence of SEQ ID NO: 2. According to an exemplary embodiment of the present invention, the target protein may be hydrophobin, more preferably a Class I hydrophobin, and still more preferably a Class I DewA, but the scope of the present invention is not limited thereto.

[0079] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the ramp tag may be obtained by collecting a rare codon of the host cell, but is not limited thereto.

[0080] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the host cell may be a bacterium belonging to *Escherichia* sp., *Salmonellae* sp., *Yersinia* sp., *Shigella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Proteus* sp., or *Klebsiella* sp, but is not limited thereto. According to an exemplary embodiment of the present invention, as a specific example, *Escherichia* sp. may be used, and more specifically, *E. coli* BL21 or the like may be used.

[0081] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, a two phase separation method using an organic solvent may be used in the purification, but the present invention is not limited thereto. Any method may be appropriately introduced as long as it is a purification method capable of separating the soluble protein according to the present invention with high efficiency.

[0082] In a case where the organic solvent is used in the purification, each of the first solvent and the second solvent may be selected from the group consisting of C3 organic solvents. Specific examples of the first solvent and the second solvent include isopropyl alcohol. In addition, according to an exemplary embodiment of the present invention, as a specific example, in a case where isopropyl alcohol is used as each of the first solvent and the second solvent and a two phase separation method is applied, a target protein expressed in a soluble form may be separated with high efficiency, which is preferable. However, the scope of the present invention is not limited thereto.

[0083] The purification of the recombinant fusion protein expressed in a soluble form by the two phase separation method according to the present invention is a new attempt that has not been applied in the related art (refer to Korean Patent Laid-Open Publication No. 2014-0022839, there have been attempts to purify hydrophobin using C₁-C₃ alcohol, but it was not a purification method for soluble expression). Thus, it was confirmed that a protein expressed in a soluble form may be separated and purified with high efficiency from specific examples to be described below. FIG. 3 illustrates an example of the method of purifying a recombinant fusion protein expressed in a soluble form by

the two phase separation method using the organic solvent according to the present invention. In FIG. 3, it is confirmed that the protein expressed in a soluble form may be purified by the above method with high efficiency.

[0084] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, processes such as recovery, suspension, and lysing of cells that are performed after the soluble expression of the target protein may be performed by appropriately introducing a reagent, method, and the like known to those skilled in the art in a range in which the object of the present invention may be achieved.

[0085] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the centrifugation in the sub-step b1) may be performed in a temperature range of 1 to 10° C., preferably 2 to 8° C., and more preferably 3 to 6° C. Since the first supernatant may be effectively separated from the lysed cell in the above range, the centrifugation is preferably performed in the above range.

[0086] In addition, the centrifugation in each of the sub-step b2) and b3) may be performed in a temperature range of 15 to 28° C., preferably 17 to 25° C., and more preferably 19 to 23° C. Since the second supernatant and the target protein may be effectively separated from the first mixture and the second mixture, respectively, in the above range, each centrifugation is preferably performed in the above range.

[0087] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the centrifugation may be performed at 3,000 to 20,000 rpm for 1 to 90 minutes. According to an exemplary embodiment of the present invention, the centrifugation in the sub-step b1) may be performed at 3,000 to 10,000 rpm, preferably 4,000 to 8,000 rpm, and more preferably 5,000 to 7,000 rpm, for 40 to 80 minutes, preferably 45 to 75 minutes, and more preferably 50 to 70 minutes, but is not limited thereto. Since the first supernatant may be effectively separated from the lysed cell in the above range, the centrifugation is preferably performed in the above range.

[0088] In addition, the centrifugation in the sub-step b2) may be performed at 6,000 to 15,000 rpm, preferably 7,500 to 13,500 rpm, and more preferably 8,500 to 11,500 rpm, for 40 to 80 minutes, preferably 45 to 75 minutes, and more preferably 50 to 70 minutes, but is not limited thereto. Since the second supernatant may be effectively separated from the first mixture in the above range, the centrifugation is preferably performed in the above range.

[0089] In addition, the centrifugation in the sub-step b3) may be performed at 6,000 to 15,000 rpm, preferably 7,500 to 13,500 rpm, and more preferably 8,500 to 11,500 rpm, for 1 to 20 minutes, preferably 4 to 16 minutes, and more

preferably 7 to 13 minutes, but is not limited thereto. Since the target protein may be effectively separated from the second supernatant in the above range, the centrifugation is preferably performed in the above range.

[0090] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, a volume ratio of the organic solvent to the first supernatant may be 1:1.5 or 1:4, preferably 1:2 or 1:3.5, and more preferably 1:2 or 1:3, but is not limited thereto. According to an exemplary embodiment of the present invention, as a specific example, the volume ratio thereof may be 1:2.5.

[0091] In addition, in the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, a volume ratio of the organic solvent to the second supernatant may be 1:0.5 or 1:1.5, preferably 1:0.6 or 1:1.4, and more preferably 1:0.8 or 1:1.2, but is not limited thereto. According to an exemplary embodiment of the present invention, as a specific example, the volume ratio thereof may be 1:1.

[0092] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the target protein may be hydrophobin, more preferably Class I hydrophobin, and still more preferably Class I DewA, and a wild type target protein may consist of an amino acid sequence of SEQ ID NO: 2. According to an exemplary embodiment of the present invention, the target protein of which the N-terminal has the mutated signal sequence may have an amino acid sequence of SEQ ID NO: 8 or 10, but the scope of the present invention is not limited thereto.

[0093] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the ramp tag may consist of 1 to 20 amino acids, preferably 6 to 12 amino acids, and more preferably 6 to 8 amino acids, but is not limited thereto. In the recombinant fusion protein according to an exemplary embodiment of the present invention, the ramp tag may consist of an amino acid sequence of SEQ ID NO: 5.

[0094] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the rare codon may be collected by analyzing a frequency of the codon and the number of isoacceptor tRNA genes.

[0095] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the frequency of the codon may be 0.05 to 3%, and preferably 0.1 to 1%, but is not limited thereto.

[0096] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the number of isoacceptor tRNA genes may be 0 to 2.

[0097] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exemplary embodiment of the present invention, the target protein may be a physiologically active protein including hormones and receptors thereof, biological response modifiers and receptors thereof, cytokines and receptors thereof, enzymes, antibodies, and antibody fragments.

[0098] In the method of purifying a recombinant fusion protein expressed in a soluble form according to an exem-

Experimental Example 1

Gene Manipulation for Recombinant Fusion Protein Expression

[0104] In order to confirm soluble expression of a target protein expressed in an insoluble inclusion body form in the related art, Class I hydrophobin DewA derived from *A. nidulans* was used as a target protein. A base sequence and an amino acid sequence thereof are shown in Table 1.

TABLE 1

Base Sequence and Amino Acid Sequence of Class I Hydrophobin DewA		
SEQ ID NO: Classification	Sequence (5' → 3')	
1 Base sequence of DewA	atgcgccttcacgtctctctctcctcgccttcactgcccgcggccaccgcaa ccgcctcccgccctctgcccgaagaacgcgaagctggccacctc ggcgcccttcgccaagcaggctgaaggcaccacctgcaatgtcggc tcgatcgcttgctgcaactccccgctgagaccaacaacgacagtctg ttgagcggtctgctcggtgctggccttctcaacgggctctcgggcaac actggcagcgctcgcgccaaggcgagcttgattgaccagctgggtct gctcgtctcgtcgaccacactgaggaaggcccgctctgcaagaaca tcgtcgcttgctgcctgaggggaaccaccaactgtgttgcgctcgaca acgctggcgccggtaccaaggtgagtaa	
2 Amino acid sequence of DewA	MRFIVSLLAFTAAATATLPASAAKNAKLATS AAFKQAEGTTCNVGSIACCNPAETNNDLS LSGLLGAGLLNLSGNTGSACAKASLIDQLG LLALVDHTEEGFVCKNIVACCPGTTNCVAV DNAGAGTKAE	
3 Signal sequence of DewA (entire base sequence) ^a	atgcgccttcacgtctctctctcctcgccttcactgcccgcggccaccgcaa ccgcctcccgccctctgcccga	

^aThe signal sequence is a sequence predicted by using SignalP (Ver 4.0) which is an exocrine signal sequence prediction program.

plary embodiment of the present invention, as a specific example, the target protein may be selected from the group consisting of hydrophobin, GST, MBP, NusA, CBP, GFP, Thioredoxin, Mistic, Sumo, and DSB, and more specifically, the target protein may be hydrophobin, but is not limited thereto.

[0099] Hereinafter, the present invention will be described in detail with reference to examples. The examples are provided only to explain the present invention in more detail, but the scope of the present invention is not limited by these examples.

Reagent, Material, and Protocol

[0100] Target protein: GenBank ID: gi67902037, Class I hydrophobin DewA (BASF, 2009) derived from *Aspergillus nidulans*, the target protein was synthesized by Bioneer Inc. (Daejeon, Korea) based on the genetic information provided by GenBank.

[0101] The ramp tag was prepared by the method of analyzing codon usage of *E. coli* described in Korean Patent Publication No. 1446054.

[0102] The pET24a plasmid vector was obtained from New England Labs (UK).

[0103] Other reagents were obtained from Sigma Aldrich (US).

A part or all of the signal sequence was deleted by using a general polymerase chain reaction (PCR) method well known to those skilled in the art.

[0105] In addition, a sequence of a ramp tag linked to an N-terminal of the Class I hydrophobin DewA used as the target protein was produced by applying the method of analyzing a rare codon disclosed in Korean Patent Publication No. 1446054. A base sequence and an amino acid sequence thereof are shown in Table 2.

TABLE 2

Base Sequence and Amino Acid Sequence of Ramp Tag, and Base Sequence of His-Tag		
SEQ ID NO: Classification	Sequence (5' → 3')	
4 Base sequence of ramp tag	cttcacagtctctaattccc	
5 Amino acid sequence of ramp tag	LPASAA	
6 Base sequence of 6 × His Tag	caccaccaccaccaccac	

[0106] An experimental group in which a part or all of the signal sequence in the base sequence of the Class I hydrophobin DewA of Table 1 was deleted was prepared, and then sequences of hydrophobin DewA of which a signal sequence was mutated were given from SEQ ID NO 7 as shown in Table 3.

TABLE 3

Mutated Hydrophobin DewA according to Signal Sequence Mutation		
SEQ ID NO:	Experimental group	Base sequence and amino acid sequence of mutated hydrophobin (5' → 3')
7	Example 1	aagaacgcgaagctggccacctcgggcgcccttcgccaagcaggctgaa ggcaccacctgcaatgtcggtcgatcgcttgctgcaactcccccgctga gaccaacaacgacagtctgttgagcggctctgctcggtgctggccttctcaa cggtctctcgggcaacactggcagcgctcgcccaaggcgagcttgatt gaccagctgggtctgctcgctctcgctcgaccacactgaggaaggcccggt ctgcaagaacatcgctcgcttgcctgaggggaaccaccaactgtgttgc cgctgacaacgctggcgccggtaccaaggctgagt aa KNAKLATSAFAKQAE GTTCNVGSIACNSPAE 8 TNND SLLSGLLGAGLLNGLSGNTGSACAKASLI DQLGLLALVDHTEEGPVCKNIVACCPEGTTNCV AVDNAGAGTKAE
9	Example 2	ctcccgccctctgcccgaagaacgcgaagctggccacctcgggcgccct tcgccaagcaggctgaaggcaccacctgcaatgtcggtcgatcgcttgc tgcaactcccccgctgagaccaacaacgacagtctgttgagcggctctgct cggtgctggccttctcaacgggctctcgggcaacactggcagcgccctgc gccaaggcgagcttgattgaccagctgggtctgctcgctctcgctcgacca cactgaggaaggcccggtctgcaagaacatcgctcgcttgcctgaggg gaaccaccaactgtgttgcgctcgacaacgctggcgccggtaccaaggc tgagtaa
10		LPASAAKNAKLATSAFAKQAE GTTCNVGSIAC CNSPAETNND SLLSGLLGAGLLNGLSGNTGSAC AKASLIDQLGLLALVDHTEEGPVCKNIVACCPE GTTNCVAVDNAGAGTKAE
1	Comparative Example 1	Atgcgcttcacgtctctctctcctcgcccttcaactgccgcggccaccgcaacc gccctcccgccctctgcccgaagaacgcgaagctggccacctcgggcg gccttcgccaagcaggctgaaggcaccacctgcaatgtcggtcgatcg cttgcctgcaactcccccgctgagaccaacaacgacagtctgttgagcgggt ctgctcggtgctggccttctcaacgggctctcgggcaacactggcagcgc ctgcgccaaggcgagcttgattgaccagctgggtctgctcgctctcgctcga ccacactgaggaaggcccggtctgcaagaacatcgctcgcttgcctg agggaaaccaccaactgtgttgcgctcgacaacgctggcgccggtaccaa ggctgagtaa

Example 1

[0107] The base sequence of SEQ ID NO: 7 obtained by deleting the entire signal sequence in the base sequence of SEQ ID NO: 1, the base sequence of SEQ ID: 4 of the ramp tag, and the sequence of SEQ ID NO: 6 of 6×His Tag were synthesized as in the order illustrated in the schematic view of FIG. 1, and then the pET24a plasmid vector was used by a general cloning method, thereby preparing a recombinant expression vector (pET24a-Ramp-DewA(-ss)-6×His).

Example 2

[0108] A recombinant expression vector was prepared in the same procedure and condition as those of Example 1, except that the base sequence of the mutated hydrophobin was changed to a base sequence of SEQ ID NO: 8.

Comparative Example 1

[0109] A recombinant expression vector (pET24a-Ramp-DewA-6×His) was prepared in the same procedure and condition as those of Example 1, except that the signal sequence (SEQ ID NO: 1) was not mutated.

Experimental Example 2

Confirmation of Soluble Expression of Recombinant Fusion Protein

[0110] In order to confirm soluble expression of the recombinant fusion protein in each experimental group of

Table 3, *E. coli* BL21(DE3) was transformed with each recombinant expression vector, and the transformed *E. coli* BL21(DE3) was inoculated into a solid LB medium (10 g/L of NaCl, 10 g/L of tryptone, 5 g/L of yeast extract) containing agar to which 100 ug/mL of kanamycin was added and then cultured in a solid state at 37° C. for 12 hours.

[0111] Thereafter, in each experimental group, three single colonies (#1, #2, and #3) were inoculated into each of 3.5 mL of a liquid LB medium and then pre-cultured at 37° C. and 220 rpm for 5 or 6 hours.

[0112] 100 uL of the pre-cultured medium was sub-cultured in 3.5 mL of a new LB medium in which 100 ug/mL of kanamycin was added and cultured at 37° C. and 220 rpm, and then, when an absorbance (OD₆₀₀) at 600 nm has reached about 0.7, 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce expression of protein.

[0113] After the addition of IPTG, each experimental group was separated into two temperature experimental groups of 37° C. and 18° C., and the expression of the Class I hydrophobin DewA used as the target protein was induced at each temperature and 250 rpm for 3 hours.

[0114] Thereafter, the target protein expressed by the following method was recovered from the *E. coli* cultured at each temperature.

[0115] a) 3.5 mL of each culture medium was centrifuged at 4° C. and 13,200 rpm for 2 minutes to obtain a precipitate, and the precipitate was suspended in 200 mM Tris-HCl (pH 8.0) and then lysed by ultrasonic waves using a pulse of 2 seconds for 20 seconds.

[0116] b) The recovered cell lysate was centrifuged at 4° C. and 13,200 rpm for 20 minutes, thereby obtaining a first supernatant from which cell debris was removed. In this case, after the cell lysing, an analysis sample for SDS-PAGE analysis (total fraction: T) was obtained, and after the centrifugation of the cell lysate, an analysis sample (soluble fraction: S) was also obtained to perform the SDS-PAGE analysis. A residual fraction after the centrifugation indicates an insoluble fraction I, and the results thereof are illustrated in FIGS. 2A and 2B.

[0117] It was confirmed from the results of FIGS. 2A and 2B that, in Comparative Example 1, when the expression was induced through IPTG and then the protein was expressed at 37° C., the expression amount (95 wt %) of the target protein DewA in the insoluble fraction I was significantly larger than that in the soluble fraction S (5 wt %).

[0118] On the other hand, it was confirmed that, in Example 1, the expression amount (7 wt %) of the target protein DewA in the insoluble fraction I was significantly reduced as compared in the soluble fraction S (93 wt %), and thus, most of the DewA protein was expressed in a soluble form.

[0119] The above results mean that the Class I hydrophobin DewA used as the target protein of the present invention was expressed in a soluble form with high efficiency by the preparation of the recombinant fusion protein according to the present invention. In addition, it was confirmed that, in particular, since high expression of 15% or more of a total protein of the *E. coli* was exhibited, when a high cell density culture was performed, 1 g/L of a soluble protein was produced.

[0120] In addition, it was confirmed that in Comparative Example 1, when the culture was performed at 18° C., the expression amount (to 100 wt %) of the target protein DewA in the insoluble fraction I was mostly occupied as compared in the soluble fraction S (hardly occupied).

[0121] On the other hand, it was confirmed that in Example 1, the expression amount (5 wt %) of the target protein DewA in the insoluble fraction I was significantly reduced as compared in the soluble fraction S (95 wt %), and thus, most of the DewA protein was expressed in a soluble form.

[0122] This is similar to the culture at 37° C., and it could be confirmed that the target protein was expressed in a soluble form by deletion of the signal sequence and fusion of the ramp tag in the recombinant fusion protein, regardless of the culture temperature.

[0123] Meanwhile, it was confirmed that in Example 2, the soluble expression of the target protein DewA was somewhat improved as compared in Comparative Example 1. However, it was evaluated that it was not applicable to the purification method of the present invention premised on the soluble expression of the target protein (data not shown).

[0124] It should be understood that it is obvious to those skilled in the art that the above results were shown by way of example only for the Class I hydrophobin DewA; however, the above results can be reasonably generalized by being extended to at least Class I hydrophobin and broadly to the whole hydrophobin, because hydrophobin has a common feature to form amphipathic structure with four disulfide bonds (S—S) in a molecule due to eight cysteines included in the molecule.

Experimental Example 3

Purification of Class I Hydrophobin DewA Expressed in Soluble Form

[0125] In each experimental group of Experimental Example 1, a recombinant fusion protein was expressed in *E. coli* in the same manner as that of Experimental Example 2, and then Class I hydrophobin DewA used as the target protein expressed in a soluble form was purified by the following purification process.

[0126] a) The Class I hydrophobin DewA was expressed in the same manner as that in each experimental group of Table 3.

[0127] b) A culture medium in which DewA was expressed was centrifuged at 4° C. and 6,000 rpm to recover cells.

[0128] c) The recovered cells were re-suspended with 200 mM Tris-HCl (pH 8.0) and were sonicated to lyse the cells. In this case, the entire fraction sample was obtained.

[0129] d) The lysed cells were centrifuged at 4° C. and 10,000 rpm for 1 hour to recover a first supernatant. In this case, a soluble fraction sample was obtained.

[0130] e) Isopropyl alcohol whose volume is greater by 2.5 times than that of the first supernatant was slowly added to the first supernatant to obtain a first mixture.

[0131] f) The first mixture was shaken at 30° C. and 200 to 250 rpm for 15 minutes.

[0132] g) After the shaking, the first mixture was centrifuged at 20° C. (room temperature) and 10,000 rpm for 10 minutes to recover a second supernatant.

[0133] h) Isopropyl alcohol whose volume is the same as that of the second supernatant was slowly added to the second supernatant to obtain a second mixture.

[0134] i) The second mixture was shaken at 30° C. and 200 to 250 rpm for 15 minutes.

[0135] j) Thereafter, after the shaking, the second mixture was centrifuged at 20° C. (room temperature) and 10,000 rpm for 10 minutes to separate Class I hydrophobin DewA protein used as the target protein purified in a white precipitated form.

[0136] Each of the total fraction sample and the soluble fraction samples obtained in c) and d) and the target protein DewA in the white precipitated form obtained in j) was dissolved in 1 mL of 200 mM Tris-HCl (pH 8.0) used as a re-suspension solvent, 1 mL of Tris-HCl was continuously and sequentially added, and then SDS-PAGE analysis of the diluted sample was performed. The results thereof are illustrated in FIG. 4.

[0137] In FIG. 4, lane 1 represents a total fraction sample T, lane 2 represents a soluble fraction sample S, lane 3 represents a sample obtained by dissolving Class I hydrophobin DewA used as a target protein purified by the purification method in 1 mL of 200 mM Tris-HCl (pH 8.0), lane 4 represents a sample obtained by additionally adding 1 mL of Tris-HCl to the sample of lane 3, and each of lanes 5 to 7 represents a diluted sample obtained by adding 1 mL of 200 mM Tris-HCl to the previous sample in the same manner as described above.

[0138] As can be seen from the above results, it is shown that 80% or more of the total fraction sample of lane 1 is also observed in the soluble fraction sample of lane 2, and, in each of lanes 3 to 7, the target protein expressed in a soluble form may be efficiently purified by the two phase separation method using the organic solvent of the present invention.

[0139] It was confirmed from the results that the hydrophobin expressed in a soluble form was efficiently purified by a simple purification method without a complex physical and chemical purification process.

[0140] In addition, it should be understood that it is obvious to those skilled in the art that, similarly to Experimental Example 2, the above results were shown by way of example only for the Class I hydrophobin DewA; however, the above results can be reasonably generalized by being extended to at least Class I hydrophobin and broadly to the whole hydrophobin, because hydrophobin has a common feature to form amphipathic structure with four disulfide bonds (S—S) in a molecule due to eight cysteines included in the molecule.

Comparative Example 2

[0141] The recombinant fusion protein expressed through Example 1 was purified in the same procedure and condition as those of Experimental Example 3, except that each of methanol, ethanol, and isobutyl alcohol was used as the organic solvent instead of the isopropyl alcohol.

Comparative Example 3

[0142] The recombinant fusion protein expressed through Example 1 was purified in the same procedure and condition as those of Experimental Example 3, except that the amount of isopropyl alcohol to be mixed with the second supernatant was set to be the same as the volume of the first supernatant.

Comparative Example 4

[0143] The recombinant fusion protein expressed through Example 1 was purified in the same procedure and condition as those of Experimental Example 3, except that a concentration of the Tris-HCl (pH 8.0) used as a re-suspension solvent was set to be 50 mM and 400 mM.

[0144] As a result, it was confirmed that, when the purification was performed by a method outside the scope of the present invention in each of Comparative Examples 2 to 4, the Class I hydrophobin DewA expressed in a soluble form was not efficiently purified (data not shown).

[0145] Therefore, it should be understood that it is obvious to those skilled in the art that the hydrophobin expressed in a soluble form may be efficiently purified only by performing the purification under the purification process of the present invention and the corresponding condition, and as described in Experimental Examples 2 and 3, the above results were shown by way of example only for the Class I hydrophobin DewA; however, the above results can be reasonably generalized by being extended to at least Class I hydrophobin and broadly to the whole hydrophobin, because hydrophobin has a common feature to form amphipathic structure with four disulfide bonds (S—S) in a molecule due to eight cysteines included in the molecule.

Comparative Example 5

Purification of Hydrophobin According to Related Art (Triton X-114 Used as Nonionic Surfactant)

[0146] A fusion protein was purified using Triton X-114 used as a nonionic surfactant by a method described in the document (Clifton N. J. et al. Article in Methods in molecular biology, 2012) including the corresponding contents.

[0147] The recombinant expression vector (pET24a-Ramp-DewA(-ss)-6×His) of Example 1 was cultured at 37° C. and 200 rpm and 0.2 mM IPTG was added at the time at which an absorbance at 600 nm has reached about 0.5 to induce expression of the recombinant fusion protein.

[0148] The expressed cells were centrifuged at 10,000 rpm to separate the medium and the cells from each other, and the recovered cells were sonicated, thereby obtaining a total fraction sample.

[0149] The total fraction sample was centrifuged at 4° C. and 10,000 rpm for 30 minutes to obtain a soluble fraction sample (supernatant).

[0150] Then, Triton X-114 used as a nonionic surfactant was added thereto in an amount of 5% of a total volume and mixed with the supernatant for 1 minute, and then the mixture was allowed to stand at room temperature to induce phase separation.

[0151] Then, only a lower part containing the hydrophobin was left by removing an upper part, isobutyl alcohol whose volume is the same as that of the lower part was added to the lower part, vortexing was performed for 1 minute to mix them, and the mixture was centrifuged at 4° C. and 5,000 rpm while confirming phase separation.

[0152] The upper part removed in the previous step was also extracted by using isobutyl alcohol in the same manner as described above.

[0153] The above procedure and results are illustrated in FIG. 5.

[0154] In FIG. 5, a tube 1 shows a state immediately after the cells are lysed, a total fraction sample 1 and a soluble fraction sample 2 after centrifugation of the total fraction sample 1 are obtained, a soluble fraction sample (supernatant) is mixed with Triton X-114, and then the mixture is subjected to vortexing.

[0155] A tube 2 shows a state after a phase is separated into an upper part 3 and a lower part 4.

[0156] A tube 3 shows a state after the upper part 3 is mixed with isobutyl alcohol and a phase of the mixture is separated into an upper part and a lower part 5. A tube 4 shows a state after the lower part 4 is mixed with isobutyl alcohol and a phase of the mixture is separated into an upper part and a lower part 6.

[0157] Results of performing two times of SDS-PAGE analysis on a total of six samples obtained in each step are illustrated in FIGS. 6 A and B.

[0158] In FIGS. 6 A and B, lane 1 represents a total fraction sample, lane 2 represents a soluble fraction sample, lane 3 represents a sample of the upper part in the tube 2, lane 4 represents a sample of the lower part in the tube 2, lane 5 represents a sample of the lower part in the tube 3, and lane 6 represents a sample of the lower part in the tube 4.

[0159] As described above, hydrophobin expressed in a soluble form to be expected in lane 6 was not observed, and the reason was that expression and purification of soluble hydrophobin were not performed by the purification method using Triton X-114 used as a nonionic surfactant according to the related art. However, it was confirmed that hydrophobin protein expressed in an insoluble inclusion body form in the related art was expressed in a soluble form, and then the hydrophobin protein was purified with high efficiency through a simple process by the configuration of the recombinant fusion protein and the purification method according to the present invention.

[0160] As set forth above, soluble expression and its simple purification of hydrophobin expressed in an inclusion body form in the related art is achieved by the soluble expression and the purification method according to the

present invention, such that protein refolding in the related art is not required, thereby easily purifying and obtaining hydrophobin expressed in a soluble form with high efficiency.

SEQUENCE LISTING

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<220> FEATURE:

<223> OTHER INFORMATION: DewA nucleotide

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ggcaccacct gcaatgtcgg ctcgatcgct tgetgcaact cccccgctga gaccaacaac      180
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ggcagcgcct gcgccaagc gagcttgatt gaccagctgg gtctgctcgc tctcgtcgac      300
cacactgagg aaggccccgt ctgcaagaac atcgtcgctt gctgcctga gggaaccacc      360
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20        25        30
Ala Ala Phe Ala Lys Gln Ala Glu Gly Thr Thr Cys Asn Val Gly Ser
35        40        45
Ile Ala Cys Cys Asn Ser Pro Ala Glu Thr Asn Asn Asp Ser Leu Leu
50        55        60
Ser Gly Leu Leu Gly Ala Gly Leu Leu Asn Gly Leu Ser Gly Asn Thr
65        70        75        80
Gly Ser Ala Cys Ala Lys Ala Ser Leu Ile Asp Gln Leu Gly Leu Leu
85        90        95
Ala Leu Val Asp His Thr Glu Glu Gly Pro Val Cys Lys Asn Ile Val
100       105       110
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<220> FEATURE:

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<400> SEQUENCE: 4

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<220> FEATURE:

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gccaaaggcga gcttgattga ccagctgggt ctgctcgtc tcgtcgacca cactgaggaa 240
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 20 25 30
 Glu Thr Asn Asn Asp Ser Leu Leu Ser Gly Leu Leu Gly Ala Gly Leu
 35 40 45
 Leu Asn Gly Leu Ser Gly Asn Thr Gly Ser Ala Cys Ala Lys Ala Ser
 50 55 60
 Leu Ile Asp Gln Leu Gly Leu Leu Ala Leu Val Asp His Thr Glu Glu
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aacaacgaca gtctgttgag cggctctgctc ggtgctggcc ttctcaacgg gctctcgggc      180
aacactggca gcgctcgcg caaggcgagc ttgattgacc agctgggtct gctcgctctc      240
gtcgaccaca ctgaggaagg ccccgctgc aagaacatcg tcgcttgctg cctgagggga      300
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 35 40 45
 Leu Leu Gly Ala Gly Leu Leu Asn Gly Leu Ser Gly Asn Thr Gly Ser
 50 55 60
 Ala Cys Ala Lys Ala Ser Leu Ile Asp Gln Leu Gly Leu Leu Ala Leu
 65 70 75 80
 Val Asp His Thr Glu Glu Gly Pro Val Cys Lys Asn Ile Val Ala Cys
 85 90 95

-continued

Cys	Pro	Glu	Gly	Thr	Thr	Asn	Cys	Val	Ala	Val	Asp	Asn	Ala	Gly	Ala
			100					105					110		
Gly	Thr	Lys	Ala	Glu											
			115												

1. A recombinant fusion protein expressed in a soluble form, comprising:

- a target protein; and
 - a ramp tag for controlling a translation rate, fused at an N-terminal of the target protein,
- wherein a signal sequence of the N-terminal of the target protein is subjected to mutation.

2. The recombinant fusion protein of claim 1, wherein the target protein is hydrophobin.

3. The recombinant fusion protein of claim 2, wherein the target protein is a Class I hydrophobin.

4. The recombinant fusion protein of claim 3, wherein the target protein is DewA.

5. The recombinant fusion protein of claim 1, wherein a wild type target protein consists of an amino acid sequence of SEQ ID NO: 2.

6. The recombinant fusion protein of claim 1, wherein the target protein consists of an amino acid sequence of SEQ ID NO: 8 or 10.

7. The recombinant fusion protein of claim 1, wherein the ramp tag consists of an amino acid sequence of SEQ ID NO: 5.

8. The recombinant fusion protein of claim 1, wherein the signal sequence consists of a base sequence of SEQ ID NO: 3.

9. The recombinant fusion protein of claim 1, wherein the mutation is one or two or more selected from the group consisting of a substitution and a deletion of a part or all of amino acids of the signal sequence and an addition of a new amino acid.

10. A base sequence encoding the recombinant fusion protein of claim 1.

11. A vector for soluble expression of the recombinant fusion protein, the base sequence of claim 10 being introduced into the vector.

12. A transformant for soluble expression of the recombinant fusion protein, the transformant being transformed with the vector of claim 11.

13. A method of purifying a recombinant fusion protein expressed in a soluble form, the method comprising:

- a) expressing a recombinant fusion protein in a soluble form by transforming a non-human host cell with a vector into which a base sequence encoding a recombinant fusion protein is introduced, the recombinant fusion protein including: a target protein; and a ramp tag for controlling a translation speed, fused at an N-terminal of the target protein; and
- b) purifying the expressed recombinant fusion protein.

14. The method of claim 13, wherein b) includes:

- b1) suspending the transformed cell and lysing and centrifuging the suspended cell to obtain a first supernatant;
- b2) shaking and then centrifuging a first mixture obtained by adding a first solvent to the first supernatant to obtain a second supernatant; and
- b3) shaking and then centrifuging a second mixture obtained by adding a second solvent to the second supernatant to recover the target protein.

15. The method of claim 13, wherein the target protein is hydrophobin.

16. The method of claim 15, wherein the target protein is Class I hydrophobin.

17. The method of claim 16, wherein the target protein is DewA.

18. The method of claim 13, wherein a wild type target protein consists of an amino acid sequence of SEQ ID NO: 2.

19. The method of claim 13, wherein the target protein consists of an amino acid sequence of SEQ ID NO: 8 or 10.

20. The method of claim 13, wherein the ramp tag is obtained by collecting a rare codon of the host cell.

21. The method of claim 13, wherein the host cell is a bacterium belonging to *Escherichia* sp., *Salmonellae* sp., *Yersinia* sp., *Shigella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Proteus* sp., or *Klebsiella* sp.

22. The method of claim 14, wherein each of the first solvent and the second solvent is isopropyl alcohol.

23. The method of claim 22, wherein a volume ratio of the isopropyl alcohol to the first supernatant is 1:2 or 1:3.

24. The method of claim 20, wherein the ramp tag consists of an amino acid sequence of SEQ ID NO: 5.

25. The method of claim 20, wherein the rare codon is collected by analyzing a frequency of the codon and a number of isoacceptor tRNA genes.

26. The method of claim 25, wherein the frequency of the codon is 0.1 to 1%.

27. The method of claim 24, wherein the number of isoacceptor tRNA genes is 0 to 2.

28. The method of claim 13, wherein the target protein is a physiologically active protein including hormones and receptors thereof, biological response modifiers and receptors thereof, cytokines and receptors thereof, enzymes, antibodies, and antibody fragments.

29. A recombinant fusion protein expressed in a soluble form, the recombinant fusion protein being purified by the method of claim 13.

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