initiation rate *in vivo* and a slower rate of 70S formation *in vitro* (NPL 4). Furthermore, the L31-deficient strain has also been reported to show lowered fidelity (NPL 4). Thus, it was known that in the translation of peptides composed of natural amino acids, ribosomes containing the L31 protein not degraded by protease 7 have greater activity than ribosomes containing the cleaved L31 protein and ribosomes not containing the L31 protein.

[Citation List]
[Non-Patent Literature]
[0006]

[NPL 1] Maini, R., Umemoto, S. & Suga, H. Ribosome-mediated synthesis of natural product-like peptides via cell-free translation. Current opinion in chemical biology 34, 44-52, doi:10.1016/j.cbpa.2016.06.006 (2016).

[NPL 2] Hartman, M. C., Josephson, K., Lin, C. W. & Szostak, J. W. An expanded set of amino acid analogs for the ribosomal translation of unnatural peptides. PloS one 2, e972, doi:10.1371/journal.pone.0000972 (2007).

[NPL 3] Ueta M, Wada C, Bessho Y, Maeda M, Wada A. Genes Cells. 2017 May;22(5):452-471. doi: 10.1111/gtc.12488. Epub 2017 Apr 10. Genes Cells. 2017

[NPL 4] Lilleorg S, Reier K, Remme J, Liiv A. J Mol Biol. 2017 Apr 7;429(7):1067-1080. doi: 10.1016/j.jmb.2017.02.015. Epub 2017 Feb 24. J Mol Biol. 2017

[NPL 5] Chadani Y, Niwa T, Izumi T, Sugata N, Nagao A, Suzuki T, Chiba S, Ito K, Taguchi H. Mol Cell. 2017 Nov 2;68(3):528-539.e5. doi: 10.1016/j.molcel.2017.10.020. Mol Cell. 2017

[Summary]
[Technical Problem]
[0007]

An objective of the present disclosure is to provide efficient methods for producing peptides comprising unnatural amino acids, and provide engineered L31 proteins and ribosomes comprising the same for use in these methods.

[Solution to Problem]
[0008]

In producing peptides comprising unnatural amino acids using a translation system, the present inventors examined whether the efficiency of mRNA translation would differ between ribosomes comprising L31 protein not degraded by protease 7 and ribosomes comprising L31 protein cleaved by protease 7. As a result, the present inventors discovered that, when

translating mRNA encoding a peptide comprising an unnatural amino acid by the Initiation Suppression (iSP) method, the use of ribosomes comprising the L31 protein cleaved by protease 7 led to an increased amount of the translated target molecule as compared to the use of ribosomes comprising the L31 protein not degraded by protease 7. The inventors also found that the relative amount of by-products can also be reduced by using ribosomes comprising the protease 7-cleaved L31 protein. These results were contrary to the facts previously known in the translation of peptides not comprising unnatural amino acids. Furthermore, the present inventors performed peptide synthesis with various magnesium ion concentrations in the translation system, and revealed that when the magnesium ion concentration is within a certain concentration range, the amount of translation is large and the proportion of by-products is low.

[0008a]

In one aspect, the present disclosure relates to a method for producing a peptide, comprising a step of translating an mRNA encoding a peptide comprising one or more types of unnatural amino acids in a translation system that comprises a ribosome comprising an engineered L31 protein, wherein the engineered L31 protein is selected from the group consisting of the proteins of (1) to (3) below:

(1) a protein comprising an amino acid sequence with deletion of 6 to 50 amino acid residues from the C terminus in the amino acid sequence set forth in SEQ ID NO: 1;

(2) a protein comprising an amino acid sequence with one or more amino acid insertions, substitutions, deletions, and/or additions in the amino acid sequence of the protein of (1); and

(3) a protein comprising an amino acid sequence having 90% or more sequence identity to the amino acid sequence of the protein of (1), and wherein the ribosome comprising the protein of (2) and (3) has greater activity for translation of the peptide comprising the unnatural amino acids, as compared to the ribosome comprising the wild-type Escherichia coli L31 that comprises the amino acid sequence of SEQ ID NO: 1.

[0008b]

In another aspect, the present disclosure relates to a method of screening for a peptide that binds to a target substance, comprising steps (a), (b) and (c) below:

(a) producing the peptide using the method as disclosed herein;

(b) contacting the target substance with the peptide or a library comprising the peptides; and

(c) selecting the peptide that binds to the target substance.

[0008c]

In another aspect, the present disclosure relates to an engineered L31 protein selected from the group consisting of proteins described in the following (1), (2) or (3):

(1) a protein comprising an amino acid sequence with deletion of 9 to 50 amino acid residues from the C terminus in the amino acid sequence set forth in SEQ ID NO: 1;

(2) a protein comprising an amino acid sequence with 1-9 amino acid insertions, substitutions, deletions, and/or additions in the amino acid sequence of the protein of (1); and
(3) a protein comprising an amino acid sequence having 90% or more sequence identity to the amino acid sequence of the protein of (1), and wherein the protein has a greater activity for translation of the peptide comprising the unnatural amino acid, as compared to a ribosome comprising the wild-type E. coli L31 that comprises the amino acid sequence of SEQ ID NO: 1.
[0008d]

In another aspect, the present disclosure relates to an isolated nucleic acid encoding the engineered L31 protein as disclosed herein. In another aspect, the present disclosure relates to a vector or cell comprising the nucleic acid as disclosed herein.
[0008e]

In another aspect, the present disclosure relates to a method for producing a ribosome comprising an engineered L31 protein, comprising steps (a) to (c) below:
(a) culturing the cell as disclosed herein;
(b) producing a lysate from culture of the cell; and
(c) purifying the ribosome from the lysate.
[0008f]

In another aspect, the present disclosure relates to a ribosome comprising the engineered L31 protein as disclosed herein. In yet another aspect, the present disclosure relates to a composition comprising the ribosome as disclosed herein.
[0008g]

In another aspect, the present disclosure relates to. a method for producing a cell-free translation system, comprising the steps of: (a) producing a ribosome by the method as disclosed herein; and (b) mixing the ribosome and an initiator tRNA acylated with an unnatural amino acid.
[0008h]

In yet another aspect, the present disclosure relates to a method for producing a cell-free translation system, comprising the following steps (a), (b) and (c):
(a) producing a lysate by disrupting a wild-type *Escherichia coli* culture under a condition where the magnesium ion concentration is 5 mM or less,
(b) purifying a ribosome from the lysate; and
(c) mixing the ribosome and an initiator tRNA acylated with an unnatural amino acid.
[0008i]

In another aspect, the present disclosure relates to a method for producing a peptide, comprising steps (i) and (ii): (i) producing a cell-free translation system using the method as disclosed herein; and (ii) translating a mRNA encoding the peptide comprising one or more types of unnatural amino acids using the cell-free translation system.

[0008i]

In another aspect, the present disclosure relates to a method for screening a peptide that can bind to a target molecule, comprising steps (I), (II) and (III):

(I) producing the peptide using the method as disclosed herein;

(II) contacting the target molecule with the peptide or a library comprising the peptide; and

(III) selecting the peptide that can bind to the target molecule.

[0009]

The present disclosure is based on such findings, and specifically relates to the following:

[1] a method for producing a peptide, comprising a step of translating an mRNA encoding a peptide comprising one or more types of unnatural amino acids in a translation system that comprises a ribosome comprising an engineered L31 protein, wherein the ribosome comprising the engineered L31 protein has greater activity for translation of the peptide comprising the unnatural amino acids, as compared to a ribosome comprising wild-type *Escherichia coli* L31 that comprises the amino acid sequence of SEQ ID NO: 1;

[2] a method for producing a peptide, comprising a step of translating an mRNA encoding a peptide comprising one or more types of unnatural amino acids in a translation system that comprises a ribosome comprising an engineered L31 protein, wherein the engineered L31 protein is selected from the group consisting of the proteins of (1) to (3) below:

(1) a protein comprising an amino acid sequence with deletion of 6 or more amino acid residues from the C terminus in the amino acid sequence set forth in SEQ ID NO: 1;

(2) a protein comprising an amino acid sequence with one or more amino acid insertions, substitutions, deletions, and/or additions in the amino acid sequence of the protein of (1); and

(3) a protein comprising an amino acid sequence having 80% or more sequence identity to the amino acid sequence of the protein of (1);

[3] a method for producing a peptide, comprising a step of translating an mRNA encoding a peptide comprising one or more types of unnatural amino acids in a translation system that comprises a ribosome comprising an engineered L31 protein, wherein the engineered L31 protein is selected from the group consisting of the proteins of (1) to (3) below:

(1) a protein consisting of an amino acid sequence with deletion of 6 or more amino acid residues from the C terminus in the amino acid sequence set forth in SEQ ID NO: 1;

(2) a protein consisting of an amino acid sequence with one or more amino acid insertions,

[CLAIMS]

1. A method for producing a peptide, comprising a step of translating an mRNA encoding a peptide comprising one or more types of unnatural amino acids in a translation system that comprises a ribosome comprising an engineered L31 protein, wherein the engineered L31 protein is selected from the group consisting of the proteins of (1) to (3) below:
(1) a protein comprising an amino acid sequence with deletion of 6 to 50 amino acid residues from the C terminus in the amino acid sequence set forth in SEQ ID NO: 1;
(2) a protein comprising an amino acid sequence with one or more amino acid insertions, substitutions, deletions, and/or additions in the amino acid sequence of the protein of (1); and
(3) a protein comprising an amino acid sequence having 90% or more sequence identity to the amino acid sequence of the protein of (1), and
wherein the ribosome comprising the protein of (2) and (3) has greater activity for translation of the peptide comprising the unnatural amino acids, as compared to the ribosome comprising the wild-type *Escherichia coli* L31 that comprises the amino acid sequence of SEQ ID NO: 1.

2. The method of claim 1, wherein the proportion of ribosomes comprising the engineered L31 protein relative to all ribosomes in the translation system is 50% or more.

3. The method of claim 1 or 2, further comprising a step of cyclizing the peptide.

4. The method of any one of claims 1 to 3, wherein the peptide comprises an unnatural amino acid at a position corresponding to its initial amino acid.

5. A method of screening for a peptide that binds to a target substance, comprising steps (a), (b) and (c) below:
(a) producing the peptide using the method of any one of claims 1 to 4;
(b) contacting the target substance with the peptide or a library comprising the peptides; and
(c) selecting the peptide that binds to the target substance.

6. An engineered L31 protein selected from the group consisting of proteins described in the following (1), (2) or (3):
(1) a protein comprising an amino acid sequence with deletion of 9 to 50 amino acid residues from the C terminus in the amino acid sequence set forth in SEQ ID NO: 1;
(2) a protein comprising an amino acid sequence with 1-9 amino acid insertions, substitutions, deletions, and/or additions in the amino acid sequence of the protein of (1); and

(3) a protein comprising an amino acid sequence having 90% or more sequence identity to the amino acid sequence of the protein of (1), and
wherein the protein has a greater activity for translation of the peptide comprising the unnatural amino acid, as compared to a ribosome comprising the wild-type *E. coli* L31 that comprises the amino acid sequence of SEQ ID NO: 1.

7. An isolated nucleic acid encoding the engineered L31 protein of claim 6 .

8. A vector or cell comprising the nucleic acid of claim 7.

9. A method for producing a ribosome comprising an engineered L31 protein, comprising steps (a) to (c) below:
(a) culturing the cell of claim 8;
(b) producing a lysate from culture of the cell; and
(c) purifying the ribosome from the lysate.

10. A ribosome comprising the engineered L31 protein of claim 6.

11. A composition comprising the ribosome of claim 10.

12. A method for producing a cell-free translation system, comprising the steps of:
(a) producing a ribosome by the method of claim 9; and
(b) mixing the ribosome and an initiator tRNA acylated with an unnatural amino acid.

13. A method for producing a cell-free translation system, comprising the following steps (a), (b) and (c):
(a) producing a lysate by disrupting a wild-type *Escherichia coli* culture under a condition where the magnesium ion concentration is 5 mM or less,
(b) purifying a ribosome from the lysate; and
(c) mixing the ribosome and an initiator tRNA acylated with an unnatural amino acid.

14. A method for producing a peptide, comprising steps (i) and (ii):
(i) producing a cell-free translation system using the method of claim 12 or 13; and
(ii) translating a mRNA encoding the peptide comprising one or more types of unnatural amino acids using the cell-free translation system.

15. A method for screening a peptide that can bind to a target molecule, comprising steps (I), (II) and (III):

(I) producing the peptide using the method of claim 14;

(II) contacting the target molecule with the peptide or a library comprising the peptide; and

(III) selecting the peptide that can bind to the target molecule.