



(86) Date de dépôt PCT/PCT Filing Date: 2004/11/04
(87) Date publication PCT/PCT Publication Date: 2005/05/19
(45) Date de délivrance/Issue Date: 2017/05/30
(85) Entrée phase nationale/National Entry: 2006/04/28
(86) N° demande PCT/PCT Application No.: JP 2004/016710
(87) N° publication PCT/PCT Publication No.: 2005/045006
(30) Priorités/Priorities: 2003/11/05 (JP2003-375823);
2004/06/25 (JP2004-189010)

(51) Cl.Int./Int.Cl. *C12N 1/21* (2006.01),
C07K 5/04 (2006.01), *C12N 15/09* (2006.01),
C12N 15/57 (2006.01), *C12P 21/00* (2006.01)
(72) Inventeurs/Inventors:
HASHIMOTO, SHIN-ICHI, JP;
TABATA, KAZUHIKO, JP
(73) Propriétaire/Owner:
KYOWA HAKKO BIO CO., LTD., JP
(74) Agent: GOUDREAU GAGE DUBUC

(54) Titre : MICRO-ORGANISMES PRODUISANT DES DIPEPTIDES ET PROCEDE DE PRODUCTION DE DIPEPTIDES
A L'AIDE DE CES MICRO-ORGANISMES
(54) Title: MICROORGANISMS PRODUCING DIPEPTIDES AND PROCESS FOR PRODUCING DIPEPTIDES USING
THE MICROORGANISMS

(57) **Abrégé/Abstract:**

The present invention provides microorganisms, such as those belonging to the genus *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces*, in which the activities of one or more peptidases and one or more peptide-transporting proteins, or the activities of three or more kinds of peptidases, are reduced or lost by mutation of genes encoding the one or more peptidases and peptide-transporting proteins, and which have the ability to form a dipeptide (a) by condensation and ligation of one or more kinds of amino acids, (b) from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, or (c) from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity. Processes for producing dipeptides using the microorganisms are also disclosed.



ABSTRACT OF THE DISCLOSURE

The present invention provides microorganisms, such as those belonging to the genus *Escherichia*, *Bacillus*,
5 *Corynebacterium* or *Saccharomyces*, in which the activities of one or more peptidases and one or more peptide-transporting proteins, or the activities of three or more kinds of peptidases, are reduced or lost by mutation of genes encoding the one or more peptidases and peptide-
10 transporting proteins, and which have the ability to form a dipeptide (a) by condensation and ligation of one or more kinds of amino acids, (b) from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, or (c) from an L-amino acid amide and an L-amino acid by
15 producing a protein having L-amino acid amide hydrolase activity. Processes for producing dipeptides using the microorganisms are also disclosed.

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

MICROORGANISMS PRODUCING DIPEPTIDES AND
PROCESS FOR PRODUCING DIPEPTIDES USING THE MICROORGANISMS

Background of the Invention

5 The present invention relates to a microorganism producing a dipeptide and a process for producing a dipeptide using the microorganism.

 Dipeptides are compounds that are important as foods, pharmaceuticals, cosmetics and the like.

10 Known methods for producing dipeptides include extraction from natural products, chemical synthesis and enzymatic methods. Extraction from natural products can be used only for producing limited kinds of dipeptides, and the productivity is low because the contents of
15 desired dipeptides in natural products are low. In the synthesis of dipeptides by the chemical synthesis methods, operations such as introduction and removal of protective groups for functional groups are necessary, and racemates are also formed. The chemical synthesis methods are thus
20 considered to be disadvantageous in respect of cost and efficiency. They are unfavorable also from the viewpoint of environmental hygiene because of the use of large amounts of organic solvents and the like.

 As to the synthesis of dipeptides by the enzymatic
25 methods, the following methods are known: a method utilizing reverse reaction of protease (J. Biol. Chem., 119, 707-720 (1937)); methods utilizing thermostable aminoacyl t-RNA synthetase (Japanese Published Unexamined Patent Application No. 146539/83, Japanese Published
30 Unexamined Patent Application No. 209991/83, Japanese Published Unexamined Patent Application No. 209992/83 and Japanese Published Unexamined Patent Application No. 106298/84); methods utilizing non-ribosomal peptide synthetase (hereinafter referred to as NRPS) (Chem. Biol.,
35 7, 373-384 (2000), FEBS Lett., 498, 42-45 (2001), U.S.

Patent No. 5,795,738 and U.S. Patent No. 5,652,116); a method utilizing D-Ala-D-Ala ligase (Biochemistry, 35, 10464-10471 (1996)); methods utilizing bacilysin synthetase (J. Ind. Microbiol., 2, 201-208 (1987) and 5 Enzyme. Microbial. Technol., 29, 400-406 (2001)); and a method utilizing L-amino-acid amide hydrolase or proline iminopeptidase (W003/010307 pamphlet).

However, the method utilizing reverse reaction of protease requires introduction and removal of protective 10 groups for functional groups of amino acids used as substrates, which causes difficulties in raising the efficiency of peptide-forming reaction and in preventing peptide-degrading reaction. The methods utilizing thermostable aminoacyl t-RNA synthetase have the defects 15 that the expression of the enzyme and the prevention of reactions forming by-products are difficult.

On the other hand, the methods utilizing NRPS, D-Ala-D-Ala ligase and bacilysin synthetase do not have the problems described above and are capable of producing 20 dipeptides having specific sequences. However, they are not efficient methods because they involve reactions requiring energy donors such as ATP.

With regard to the method utilizing L-amino-acid amide hydrolase or proline iminopeptidase, it is disclosed 25 that dipeptides can be produced by using a culture of a microorganism producing the enzyme, microorganism cells isolated from the culture or a treated matter of the microorganism cells. However, the amounts of dipeptides produced by this method are not sufficient.

30 Living cells have metabolic systems in which unnecessary proteins are decomposed to constituent amino acids and the formed amino acids are used for synthesis of necessary proteins. As this function is essential for the survival and proliferation of cells, it is known that many 35 kinds of proteases and peptidases exist in living organisms.

For example, it is known that in Escherichia coli, there exist at least seven kinds of dipeptidases as well as other proteases and peptidases capable of decomposing dipeptides (FEMS Microbial. Rev., 63, 265-276 (1989)). On
5 the chromosomal DNAs of Corynebacterium glutamicum, Bacillus subtilis and Saccharomyces cerevisiae, there exist 25 or more, 14 or more, and 20 or more, respectively, genes identified as peptidase gene.

However, it is not known that introduction of
10 peptidase deficiency into a microorganism producing a dipeptide increases the production of the dipeptide.

It is also known that living cells have plural peptide-incorporating systems. For example, Escherichia coli is reported to have three systems for incorporating
15 dipeptides (Chem. Biol., 5, 489-504 (1998) and Microbiol., 145, 2891-2901 (1999)). Further, it has been confirmed from genomic DNA information that microorganisms belonging to the genera Escherichia, Bacillus, Corynebacterium and Saccharomyces all have three or more kinds of peptide-
20 incorporating systems.

However, it is not known whether dipeptides synthesized in cells are discharged outside the cells or not. Still less is known that the production of a dipeptide by a dipeptide-producing microorganism is
25 increased by introducing deficiency of a protein involved in the peptide incorporation into the microorganism.

An object of the present invention is to provide a microorganism producing a dipeptide and a process for producing a dipeptide using the microorganism.

30

Summary of the Invention

The present invention relates to the following (1) to (21).

- (1) A microorganism in which the activities of one or
35 more kinds of peptidases and one or more kinds of proteins having peptide-transporting activity

(hereinafter referred to also as peptide-transporting proteins) are reduced or lost and which has the ability to produce a dipeptide.

- 5 (2) A microorganism in which the activities of three or more kinds of peptidases are reduced or lost and which has the ability to produce a dipeptide.
- 10 (3) The microorganism according to the above (1) or (2), wherein the peptidase is a protein having the amino acid sequence shown in any of SEQ ID NOS: 1 to 4, or a protein having an amino acid sequence which has 80% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 1 to 4 and having peptidase activity.
- 15 (4) The microorganism according to the above (1) or (3), wherein the peptide-transporting protein is a protein having the amino acid sequence shown in any of SEQ ID NOS: 5 to 9, or a protein having an amino acid sequence which has 80% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 5 to 9 and having peptide-transporting activity.
- 20 (5) The microorganism according to any of the above (1) to (4), wherein the microorganism which has the ability to produce a dipeptide is a microorganism having the ability to produce a protein according to any of [1] to [4] below:
- 25 [1] a protein having the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;
- 30 [2] a protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide;
- 35 [3] a protein consisting of an amino acid sequence which has 65% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide;

and

[4] a protein having an amino acid sequence which has 80% or more homology to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide.

- 5
- (6) The microorganism according to any of the above (1) to (4), wherein the microorganism which has the ability to produce a dipeptide is a microorganism carrying DNA according to any of [1] to [4] below:
- 10 [1] DNA encoding the protein according to any of [1] to [4] of the above (5);
- [2] DNA having the nucleotide sequence shown in any of SEQ ID NOS: 26 to 32, 64 and 65;
- [3] DNA which hybridizes with DNA having the
- 15 nucleotide sequence shown in any of SEQ ID NOS: 26 to 32, 64 and 65 under stringent conditions and which encodes a protein having the activity to synthesize a dipeptide; and
- [4] DNA having a nucleotide sequence which has 80% or
- 20 more homology to the nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide.
- (7) The microorganism according to any of the above (1) to (6), wherein the microorganism which has the
- 25 ability to produce a dipeptide is a microorganism carrying a recombinant DNA in which the DNA according to any of [1] to [4] of the above (6) is ligated to a vector DNA.
- (8) The microorganism according to any of the above (1) to (7), wherein the microorganism is a microorganism
- 30 belonging to the genus Escherichia, Bacillus, Corynebacterium or Saccharomyces.
- (9) The microorganism according to any of the above (1) to (4), wherein the microorganism which has the
- 35 ability to produce a dipeptide is a microorganism having the ability to form a dipeptide from an L-

amino acid ester and an L-amino acid.

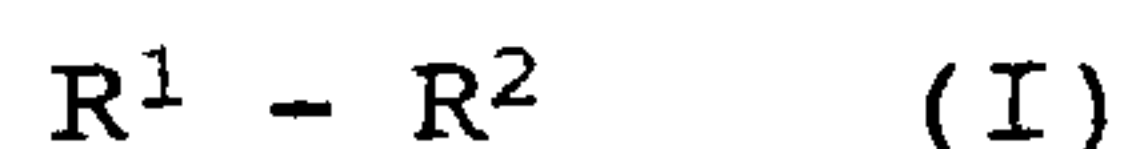
- 5 (10) The microorganism according to the above (9), wherein the microorganism having the ability to form a dipeptide from an L-amino acid ester and an L-amino acid is a microorganism producing proline iminopeptidase.
- 10 (11) The microorganism according to the above (9) or (10), wherein the microorganism having the ability to form a dipeptide from an L-amino acid ester and an L-amino acid is a microorganism carrying a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA.
- 15 (12) The microorganism according to any of the above (9) to (11), wherein the microorganism is a microorganism belonging to the genus Escherichia, Bacillus, Corynebacterium or Saccharomyces.
- 20 (13) The microorganism according to any of the above (1) to (4), wherein the microorganism is a microorganism having the ability to form a dipeptide from an L-amino acid amide and an L-amino acid.
- 25 (14) The microorganism according to the above (13), wherein the microorganism having the ability to form a dipeptide from an L-amino acid amide and an L-amino acid is a microorganism producing a protein having L-amino acid amide hydrolase activity.
- 30 (15) The microorganism according to the above (13), wherein the microorganism having the ability to form a dipeptide from an L-amino acid amide and an L-amino acid is a microorganism carrying a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA.
- 35 (16) The microorganism according to any of the above (13) to (15), wherein the microorganism is a microorganism belonging to the genus Escherichia, Bacillus, Corynebacterium or Saccharomyces.
- (17) A process for producing a dipeptide, which comprises:

allowing an enzyme source and one or more kinds of amino acids to be present in an aqueous medium, said enzyme source being a culture of the microorganism according to any of the above (1) to (8) or a treated matter of the culture;

allowing the dipeptide to form and accumulate in the aqueous medium; and

recovering the dipeptide from the medium.

(18) The process according to the above (17), wherein the dipeptide is a dipeptide represented by the following formula (I):



(wherein R^1 and R^2 , which may be the same or different, each represent an amino acid).

(19) A process for producing a dipeptide, which comprises: allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being a culture of the microorganism according to any of the above (9) to (12) or a treated matter of the culture; allowing the dipeptide to form and accumulate in the aqueous medium; and

recovering the dipeptide from the medium.

(20) A process for producing a dipeptide, which comprises: allowing an enzyme source, an L-amino acid amide and an L-amino acid to be present in an aqueous medium, said enzyme source being a culture of the microorganism according to any of the above (13) to (16) or a treated matter of the culture; allowing the dipeptide to form and accumulate in the aqueous medium; and

recovering the dipeptide from the medium.

(21) The process according to any of the above (17) to (20), wherein the treated matter of the culture is

concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, treatment with a surfactant, ultrasonication,
5 mechanical friction, treatment with a solvent, enzymatic treatment or immobilization.

The present invention also relates to a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by
10 introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP,
15 PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, YwaD, OCT1, SPC2, SPY2, YIM1, or a peptidase comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB97732, BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 or
20 BAB99819;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF,
25 AppA, AppB, AppC, AppD, AppF, YclF, Ykfd, OPT1, OPT2, PTR2, or a peptide-transporting protein comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB99048, BAB99383, BAB99384, BAB99385, BAB99713, BAB99714, BAB99715, BAB99830, BAB99831 or BAB99832;

30 wherein said microorganism belongs to the genus *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces* and has the ability:

8a

(a) to form a dipeptide by condensation and ligation of one or more kinds of amino acids by producing a protein according to any of [1] to [4] below:

5 [1] a protein comprising the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68, and which has the activity to synthesize a
10 dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

15 [4] a protein comprising an amino acid sequence which has 80% or more identity to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

(b) to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase; or
20

(c) to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity.

The present invention also relates to a microorganism in
25 which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA,
30 AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, YwaD, OCT1, SPC2, SPY2, YIM1, or a peptidase comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB97732,

8b

BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 or BAB99819;

wherein said microorganism belongs to the genus *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces* and

5 has the ability:

(a) to form a dipeptide by condensation and ligation of one or more kinds of amino acids by producing a protein according to any of [1] to [4] below:

10 [1] a protein comprising the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68, and which has the activity to synthesize a
15 dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

20 [4] a protein comprising an amino acid sequence which has 80% or more identity to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

(b) to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase; or

(c) to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity.

The present invention also relates to a microorganism in
30 which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and

8c

peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, YwaD, OCT1, SPC2, SPY2, YIM1, or a peptidase comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB97732, BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 or BAB99819;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, Ykfd, OPT1, OPT2, PTR2, or a peptide-transporting protein comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB99048, BAB99383, BAB99384, BAB99385, BAB99713, BAB99714, BAB99715, BAB99830, BAB99831 or BAB99832;

wherein said microorganism belongs to the genus *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces* and has the ability:

(a) to form a dipeptide by condensation and ligation of one or more kinds of amino acids by producing a protein according to any of [1] to [4] below:

[1] a protein comprising the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the amino acid sequence shown in

8d

any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

[4] a protein comprising an amino acid sequence which has 80% or more identity to the amino acid sequence shown in

5 SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

(b) to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase; or

(c) to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity.

The present invention also relates to a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the
15 chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, YwaD, OCT1, SPC2, SPY2, YIM1, or a peptidase comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB97732, BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 or BAB99819;

25 wherein said microorganism belongs to the genus *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces* and has the ability:

(a) to form a dipeptide by condensation and ligation of one or more kinds of amino acids by producing a protein according to
30 any of [1] to [4] below:

[1] a protein comprising the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;

8e

[2] a variant of the protein of [1] in which one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

[4] a protein comprising an amino acid sequence which has 80% or more identity to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

(b) to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase; or

(c) to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity.

The present invention also relates to a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, YwaD, OCT1, SPC2, SPY2, YIM1, or a peptidase comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB97732, BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 or BAB99819;

wherein said peptide-transporting protein is DppA, DppB,

8f

DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, YkfD, OPT1, OPT2, PTR2, or
 5 a peptide-transporting protein comprising the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB99048, BAB99383, BAB99384, BAB99385, BAB99713, BAB99714, BAB99715, BAB99830, BAB99831 or BAB99832;

wherein said microorganism belongs to the genus
 10 *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces* and has the ability:

(a) to form a dipeptide by condensation and ligation of two amino acids which are identical or different by producing a protein according to any one of [1] to [4] below:

- 15 [1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOS: 19 to 25 and 68;
 [2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOS: 19 to 25 and 68,
 20 and which has the activity to synthesize a dipeptide;
 [3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino acid sequence shown in any one of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and
 25 [4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

wherein said microorganism comprises a recombinant DNA
 30 according to any one of [i] to [iv] below, ligated to a vector DNA:

[i] DNA encoding the protein defined in any one of [1] to [4] above;

8g

[ii] DNA comprising the nucleotide sequence shown in any one of SEQ ID NOS: 26 to 32, 64 and 65;

[iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOS: 26 to 32, 64 and 65 under stringent conditions comprising hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1 to 2-fold SSC solution, and which encodes a protein having the activity to synthesize a dipeptide; and

[iv] DNA comprising a nucleotide sequence which has 80% or more identity to the full length nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide;

(b) to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA; or

(c) to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA.

The present invention also relates to a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, YwaD, OCT1, SPC2, SPY2, YIM1, or a peptidase comprising

8h

the amino acid sequence set forth in DNA Data Bank of Japan registration Nos. BAB97732, BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 or BAB99819;

wherein said microorganism belongs to the genus

5 *Escherichia*, *Bacillus*, *Corynebacterium* or *Saccharomyces* and has the ability:

(a) to form a dipeptide by condensation and ligation of two amino acids which are identical or different by producing a protein according to any one of [1] to [4] below:

10 [1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOS: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOS: 19 to 25 and 68,
15 and which has the activity to synthesize a dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino acid sequence shown in any one of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

20 [4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

wherein said microorganism comprises a recombinant DNA
25 according to any one of [i] to [iv] below, ligated to a vector DNA:

[i] DNA encoding the protein defined in any one of [1] to [4] above;

[ii] DNA comprising the nucleotide sequence shown in any one
30 of SEQ ID NOS: 26 to 32, 64 and 65;

[iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOS: 26 to 32, 64 and 65 under stringent conditions comprising

hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1 to 2-fold SSC solution, and which encodes a protein having the activity to synthesize a dipeptide; and

5 [iv] DNA comprising a nucleotide sequence which has 80% or more identity to the nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide;

(b) to form a dipeptide from an L-amino acid ester and an L-
10 amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA; or

(c) to form a dipeptide from an L-amino acid amide and an L-
15 amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA.

In accordance with the present invention, there are
20 provided a microorganism producing a dipeptide and a process for producing a dipeptide using the microorganism.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source and one or more kinds of amino
25 acids to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide
30 deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a

8j

corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, or YkfD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide by condensation and ligation of two amino acids which are identical or different by producing a protein according to any one of [1] to [4] below:

- [1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68;
- [2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;
- [3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and
- [4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

wherein said microorganism comprises a recombinant DNA according to any one of [i] to [iv] below, ligated to a vector DNA:

- [i] DNA encoding the protein defined in any one of [1] to [4] above;
- [ii] DNA comprising the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65;

8k

[iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65 under stringent conditions comprising hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1 to 2-fold SSC solution, and which encodes a protein having the activity to synthesize a dipeptide; and

[iv] DNA comprising a nucleotide sequence which has 80% or more identity to the full length nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and
- recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source and one or more kinds of amino acids to be present in an aqueous medium, said enzyme source being

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide by condensation and ligation of two amino acids which are

identical or different by producing a protein according to any one of [1] to [4] below:

- [1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68;
- 5 [2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;
- 10 [3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and
- 15 [4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;
- wherein said microorganism comprises a recombinant DNA according to any one of [i] to [iv] below, ligated to a vector
- 20 DNA:
- [i] DNA encoding the protein defined in any one of [1] to [4] above;
- [ii] DNA comprising the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65;
- 25 [iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65 under stringent conditions comprising hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1
- 30 to 2-fold SSC solution, and which encodes a protein having the activity to synthesize a dipeptide; and
- [iv] DNA comprising a nucleotide sequence which has 80% or more identity to the full length nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the
- 35 activity to synthesize a dipeptide;

or

8m

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, treatment with a surfactant, ultrasonication, mechanical friction, treatment with a solvent, enzymatic treatment or immobilization;
- allowing the dipeptide to form and accumulate in the aqueous medium; and
- recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source and one or more kinds of amino acids to be present in an aqueous medium, said enzyme source being

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide by condensation and ligation of two amino acids which are identical or different by producing a protein according to any one of [1] to [4] below:

[1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;

8n

[3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

5 [4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

wherein said microorganism comprises a recombinant DNA
10 according to any one of [i] to [iv] below, ligated to a vector DNA:

[i] DNA encoding the protein defined in any one of [1] to [4] above;

[ii] DNA comprising the nucleotide sequence shown in any
15 one of SEQ ID NOs: 26 to 32, 64 and 65;

[iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65 under stringent conditions comprising hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1 to 2-fold SSC solution, and which encodes a protein having the activity to
20 synthesize a dipeptide; and

[iv] DNA comprising a nucleotide sequence which has 80% or more identity to the full length nucleotide sequence shown
25 in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture,
30 cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

35 - recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being

(A) a culture of a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, or Ykfd;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

- recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

8p

- allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, treatment with a surfactant, ultrasonication, mechanical friction, treatment with a solvent, enzymatic treatment or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

- recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or

8q

addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

5 wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

 wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA;

 or

15 (B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

20 - allowing the dipeptide to form and accumulate in the aqueous medium; and

 - recovering the dipeptide from the medium.

 The present invention also relates to a process for producing a dipeptide, which comprises:

25 - allowing an enzyme source, an L-amino acid amide and an L-amino acid to be present in an aqueous medium, said enzyme source being:

 (A) a culture of a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

35

8r

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, or YkfD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and
- recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid amide and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

8s

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus
5 *Escherichia* and has the ability to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is
10 ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product
15 obtained by subjecting the cells to drying, freeze-drying, treatment with a surfactant, ultrasonication, mechanical friction, treatment with a solvent, enzymatic treatment or immobilization;
- allowing the dipeptide to form and accumulate in the aqueous
20 medium; and

- recovering the dipeptide from the medium.

The present invention also relates to a process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid amide and an
25 L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or
30 addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;
35

8t

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said
5 microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated
10 matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the
15 aqueous medium; and

- recovering the dipeptide from the medium.

Brief Description of the Drawings

Fig. 1 shows the steps for constructing His-tagged ywfE gene expression vector pQE60ywfE.

20 Fig. 2 shows the steps for constructing ywfE gene expression-enhanced vector pPE56.

Explanation of Symbols

PT5: T5 promoter

25 P_{trp}: Tryptophan promoter

Detailed Description of the Invention

The present invention is described in detail below.

1. Microorganisms of the Present Invention

30 The microorganisms of the present invention are microorganisms in which the activities of one or more kinds of peptidases and one or more kinds of proteins having peptide-transporting activity (hereinafter referred to as peptide-transporting proteins) are reduced or lost and which have the
35 ability to produce a dipeptide, or microorganisms in which the

8u

activities of three or more kinds of peptidases are reduced or lost and which have the ability to produce a dipeptide.

The microorganisms in which the activities of one or more kinds of peptidases and one or more kinds of peptide-
5 transporting proteins are reduced or lost include

microorganisms in which the activities of one or more arbitrary kinds of peptidases and one or more arbitrary kinds of peptide-transporting proteins are reduced or lost provided that the microorganisms can normally grow,
5 specifically, microorganisms in which the activities of preferably one to nine kinds, more preferably one to seven kinds, further preferably one to four kinds of peptidases and preferably one to five kinds, more preferably one to three kinds, further preferably one or two kinds,
10 particularly preferably one kind of peptide-transporting protein are reduced or lost.

Examples of such microorganisms are microorganisms in which the activities of one or more kinds of peptidases and one or more kinds of peptide-transporting proteins are
15 reduced or lost because the nucleotide sequences of one or more kinds of genes encoding peptidases (hereinafter referred to as peptidase genes) and one or more kinds of genes encoding peptide-transporting proteins (hereinafter referred to as peptide-transporting protein genes) among
20 the peptidase genes and peptide-transporting protein genes existing on the genomic DNA of the microorganisms are entirely or partially deleted or said nucleotide sequences contain nucleotide substitutions or additions.

The expression "the activity of peptidase is
25 reduced" means that the peptide-degrading activity is reduced compared with peptidase having none of the above deletions, substitutions and additions of nucleotides.

The peptide-degrading activity of a microorganism can be measured by allowing a peptide as a substrate and
30 microorganism cells to be present in an aqueous medium, thereby performing peptide-degrading reaction, and then determining the amount of the remaining peptide by a known method, e.g., HPLC analysis.

The above peptidases may be any proteins having
35 peptide-degrading activity. Preferred are proteins having high dipeptide-hydrolyzing activity. More preferred are

dipeptidases.

Examples of peptidases include: those existing in Escherichia coli such as PepA having the amino acid sequence shown in SEQ ID NO: 1, PepB having the amino acid sequence shown in SEQ ID NO: 2, PepD having the amino acid sequence shown in SEQ ID NO: 3, PepN having the amino acid sequence shown in SEQ ID NO: 4, PepP [GenBank accession No. (hereinafter abbreviated as Genbank) AAC75946], PepQ (GenBank AAC76850), PepE (GenBank AAC76991), PepT (GenBank AAC74211), Dcp (GenBank AAC74611) and IadA (GenBank AAC77284); those existing in Bacillus subtilis such as AmpS (GenBank AF012285), PepT (GenBank X99339), YbaC (GenBank Z99104), YcdD (GenBank Z99105), YjbG (GenBank Z99110), YkvY (GenBank Z99111), YqjE (GenBank Z99116) and YwaD (GenBank Z99123); those existing in Corynebacterium glutamicum such as proteins having the amino acid sequences represented by BAB97732, BAB97858, BAB98080, BAB98880, BAB98892, BAB99013, BAB99598 and BAB99819 (registration Nos. of DNA Data Bank of Japan); and those existing in Saccharomyces cerevisiae such as OCT1 (GenBank NC_001143), SPC2 (GenBank NC_003143), SPY2 [Saccharomyces genome database (<http://www.yeastgenome.org/>) accession No. L0002875] and YIM1 (GenBank NC_001145). Examples of dipeptidases include PepA, PepB, PepD and PepN having the amino acid sequences shown in SEQ ID NOS: 1 to 4, PepQ, PepE and IadA. Proteins having amino acid sequences which have 80% or more, preferably 90% or more, more preferably 95% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 1 to 4 and having peptidase activity are also included in the proteins having high dipeptide-degrading activity.

The homology among amino acid sequences and nucleotide sequences can be determined by using algorithm BLAST by Karlin and Altschul [Proc. Natl. Acad. Sci. USA, 90, 5873 (1993)] and FASTA [Methods Enzymol., 183, 63 (1990)]. On the basis of the algorithm BLAST, programs

such as BLASTN and BLASTX have been developed [J. Mol. Biol., 215, 403 (1990)]. When a nucleotide sequence is analyzed by BLASTN on the basis of BLAST, the parameters, for instance, are as follows: score=100 and wordlength=12.

5 When an amino acid sequence is analyzed by BLASTX on the basis of BLAST, the parameters, for instance, are as follows: score=50 and wordlength=3. When BLAST and Gapped BLAST programs are used, default parameters of each program are used. The specific techniques for these

10 analyses are known (<http://www.ncbi.nlm.nih.gov>).

The expression "the activity of a peptide-transporting protein is reduced" means that the peptide-transporting activity is reduced compared with a peptide-transporting protein having none of the above deletions, substitutions and additions of nucleotides.

15

The peptide-transporting activity of a microorganism can be measured by allowing a peptide as a substrate and microorganism cells to be present in an aqueous medium, thereby performing peptide-transporting reaction, and then

20 determining the amount of the remaining peptide by a known method, e.g., HPLC analysis.

The above peptide-transporting proteins may be any proteins involved in peptide transporation of microorganisms, for example, proteins encoded by genes

25 forming an operon on chromosomal DNA which form a complex on cell membrane to express dipeptide-transporting activity and those which have peptide-transporting activity as individual proteins. Preferred are proteins having high peptide-transporting activity.

30 Examples of the peptide-transporting proteins include: those existing in Escherichia coli such as DppA having the amino acid sequence shown in SEQ ID NO: 5, DppB having the amino acid sequence shown in SEQ ID NO: 6, DppC having the amino acid sequence shown in SEQ ID NO: 7, DppD

35 having the amino acid sequence shown in SEQ ID NO: 8, DppF having the amino acid sequence shown in SEQ ID NO: 9, OppA

(GenBank AAC76569), OppB (GenBank AAC76568), OppC (GenBank AAC76567), OppD (GenBank AAC76566), OppF (GenBank AAC76565), YddO (GenBank AAC74556), YddP (GenBank AAC74557), YddQ (GenBank AAC74558), YddR (GenBank AAC74559), YddS (GenBank AAC74560), YbiK (GenBank AAC73915), MppA (GenBank AAC74411), SapA (GenBank AAC74376), SapB (GenBank AAC74375), SapC (GenBank AAC74374), SapD (GenBank AAC74373) and SapF (GenBank AAC74372); those existing in Bacillus subtilis such as DppA (GenBank CAA40002), DppB (GenBank CAA40003), DppC (GenBank CAA40004), DppD (GenBank CAA40005), DppE (GenBank CAA40006), OppA (GenBank CAA39787), OppB (GenBank CAA39788), OppC (GenBank CAA39789), OppD (GenBank CAA39790), OppF (GenBank CAA39791), AppA (GenBank CAA62358), AppB (GenBank CAA62359), AppC (GenBank CAA62360), AppD (GenBank CAA62356), AppF (GenBank CAA62357), YclF (GenBank CAB12175) and Ykfd (GenBank CAB13157); those existing in Corynebacterium glutamicum such as proteins having the amino acid sequences represented by BAB99048, BAB99383, BAB99384, BAB99385, BAB99713, BAB99714, BAB99715, BAB99830, BAB99831 and BAB99832 (registration Nos. of DNA Data Bank of Japan); and those existing in Saccharomyces cerevisiae such as OPT1 (GenBank NP_012323), OPT2 (GenBank NP_015520) and PTR2 (GenBank CAA82172). Examples of the proteins having high peptide-transporting activity include DppA, DppB, DppC, DppD and DppF having the amino acid sequences shown in SEQ ID NOS: 5 to 9, and proteins having amino acid sequences which have 80% or more, preferably 90% or more, more preferably 95% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 5 to 9.

The homology among amino acid sequences can be determined by using programs such as BLAST and FASTA described above.

The microorganisms in which the activities of three or more kinds of peptidases are reduced or lost include

microorganisms in which the activities of three or more arbitrary kinds of peptidases are reduced or lost provided that the microorganisms can normally grow, specifically, microorganisms in which the activities of preferably three to nine kinds, more preferably three to six kinds, further preferably three or four kinds of peptidases are reduced or lost.

Examples of peptidases include the above-described peptidases and dipeptidases existing in Escherichia coli,
10 Bacillus subtilis, Corynebacterium glutamicum and Saccharomyces cerevisiae. Proteins consisting of amino acid sequences which have 80% or more, preferably 90% or more, more preferably 95% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 1 to 4 and
15 having peptidase activity are also included in the proteins having high dipeptide-degrading activity.

The homology among amino acid sequences can be determined by using programs such as BLAST and FASTA described above.

20 There is not any specific restriction as to the microorganism having the ability to produce a dipeptide so long as it has the ability to produce a dipeptide. Suitable microorganisms include microorganisms producing proteins having the activity to synthesize a dipeptide by
25 condensation and ligation of one or more kinds of amino acids, microorganisms producing proteins having the activity to synthesize a dipeptide from an L-amino acid ester and an L-amino acid, and microorganisms producing proteins having the activity to synthesize a dipeptide
30 from an L-amino acid amide and an L-amino acid.

The microorganisms producing proteins having the activity to synthesize a dipeptide by condensation and ligation of one or more kinds of amino acids include microorganisms producing a protein selected from the group
35 consisting of NRPS, D-Ala-D-Ala ligase and bacilysin synthetase.

Examples of the microorganisms producing NRPS include procaryotes such as microorganisms of the genus Bacillus, eucaryotes such as microorganisms of the genus Penicillium, microorganisms producing BacA, BacB and BacC (GenBank AF007865), microorganisms producing TycA, TycB and TycC (GenBank AF004835), microorganisms producing PcbAB (GenBank M57425), and microorganisms producing a protein having an amino acid sequence which has 80% or more, preferably 90% or more, more preferably 95% or more homology to the amino acid sequence of any protein selected from BacA, BacB, BacC, TycA, TycB, TycC and PcbAB and having NRPS activity.

Examples of the microorganisms producing D-Ala-D-Ala ligase include procaryotes forming peptidoglycans, microorganisms producing DdlA (GenBank accession No. M58467), microorganisms producing DdlB (GenBank accession No. AE000118), microorganisms producing DdlC (GenBank accession No. D88151), and microorganisms producing a protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence of any protein selected from DdlA, DdlB and DdlC and having D-Ala-D-Ala ligase activity.

The homology among amino acid sequences can be determined by using programs such as BLAST and FASTA described above.

Examples of the microorganisms producing bacilysin synthetase include microorganisms belonging to the genus Bacillus, preferably, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium and Bacillus pumilus, and microorganisms producing a protein selected from the following [1] to [4]:

- [1] a protein having the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;
- [2] a protein consisting of an amino acid sequence wherein

one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide;

5 [3] a protein consisting of an amino acid sequence which has 65% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

10 [4] a protein having an amino acid sequence which has 80% or more homology to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide.

The microorganisms producing proteins having the activity to synthesize a dipeptide from an L-amino acid ester and an L-amino acid include microorganisms producing proline iminopeptidase, specifically, those belonging to the genera Bacillus, Corynebacterium and Pseudomonas. Examples of such microorganisms are Bacillus subtilis ATCC 6633, Bacillus coagulans EK01 [J. Bacteriol., 174, 7919 (1992)], Corynebacterium glutamicum ATCC 13286, Pseudomonas putida AJ-2402 (FERM BP-8101), Pseudomonas putida ATCC 12633 and Pseudomonas putida AJ-2048 (FERM BP-8123) (microorganisms described in WO03/010307). The microorganisms producing proline iminopeptidase also include Arthrobacter nicotianae [FEMS Microbiol. Lett., 78, 191 (1999)], Escherichia coli (Japanese Published Unexamined Patent Application No. 113887/90), Flavobacterium meningosepticum [Arch. Biochem. Biophys., 336, 35 (1996)], Hafnia alvei [J. Biochem., 119, 468 (1996)], Lactobacillus delbrueckii [Microbiology, 140, 527 (1994)], Bacillus coagulans [J. Bacteriol., 174, 7919 (1994)], Aeromonas sobria [J. Biochem., 116, 818 (1994)], Xanthomonas campestris (Japanese Published Unexamined Patent Application No. 121860/97), Neisseria gonorrhoeae [Mol. Microbiol., 9, 1203 (1993)], Propionibacterium freudenreichii [Appl. Environ. Microbiol., 64, 4736

(1998)], Serratia marcescens [J. Biochem., 122, 601 (1997)], Corynebacterium variabilis [J. Appl. Microbiol., 90, 449 (2001)], Thermoplasma acidophilum [FEBS Lett., 398, 101 (1996)] and Pseudomonas aeruginosa [Nature, 406, 959 (2000)].

Further, the microorganisms producing proline iminopeptidase include microorganisms having the ability to produce a protein selected from the following [1] to [3]:

- [1] proline iminopeptidase described in any of WO03/010307; FEMS Microbiol. Lett., 78, 191 (1999); Japanese Published Unexamined Patent Application No. 113887/90; Arch. Biochem. Biophys., 336, 35 (1996); J. Biochem., 119, 468 (1996); Microbiology, 140, 527 (1994); J. Bacteriol., 174, 7919 (1994); J. Biochem., 116, 818 (1994); Japanese Published Unexamined Patent Application No. 121860/97; Mol. Microbiol., 9, 1203 (1993); Appl. Environ. Microbiol., 64, 4736 (1998); J. Biochem., 122, 601 (1997); FEBS Lett., 398, 101 (1996); and Nature, 406, 959 (2000);
- [2] a protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence of any proline iminopeptidase of the above [1] and having proline iminopeptidase activity; and
- [3] a protein consisting of an amino acid sequence which has 80% or more homology to the amino acid sequence of any proline iminopeptidase of the above [1] and having proline iminopeptidase activity.

The microorganisms producing proteins having the activity to synthesize a dipeptide from an L-amino acid amide and an L-amino acid include microorganisms producing L-amino acid amide hydrolase, specifically, those belonging to the genera Bacillus, Corynebacterium, Erwinia, Rhodococcus, Chryseobacterium, Micrococcus, Pseudomonas, Cryptococcus, Trichosporon, Rhodospiridium, Sporobolomyces,

Tremella, Torulaspora, Sterigmatomyces and Rhodotolura. Preferred are microorganisms belonging to the genera Bacillus, Corynebacterium and Pseudomonas. More preferred examples are Bacillus megaterium AJ3284 (FERM BP-8090),
5 Corynebacterium glutamicum ATCC 13286, Micrococcus luteus ATCC 9341 and Pseudomonas saccharophila ATCC 15946 (microorganisms described in WO03/010187).

The microorganisms producing proteins having L-amino acid amide hydrolase activity include microorganisms
10 having the ability to produce a protein of the following [1] or [2]:

[1] a protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence of L-amino acid amide
15 hydrolase described in WO03/010187 and having L-amino acid amide hydrolase activity;

[2] a protein consisting of an amino acid sequence which has 80% or more homology to the amino acid sequence of L-amino acid amide hydrolase described in WO03/010187 and
20 having L-amino acid amide hydrolase activity.

The above protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added and having the activity to synthesize a dipeptide can be obtained, for example, by
25 introducing a site-directed mutation into DNA encoding a protein selected from a protein consisting of the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68, a protein having proline iminopeptidase activity and a protein having L-amino acid amide hydrolase activity by
30 site-directed mutagenesis described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as Molecular Cloning, Second Edition); Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997)
35 (hereinafter referred to as Current Protocols in Molecular Biology); Nucleic Acids Research, 10, 6487 (1982); Proc.

Natl. Acad. Sci. USA, 79, 6409 (1982); Gene, 34, 315 (1985); Nucleic Acids Research, 13, 4431 (1985); Proc. Natl. Acad. Sci. USA, 82, 488 (1985), etc.

The number of amino acid residues which are deleted, substituted or added is not specifically limited, but is within the range where deletion, substitution or addition is possible by known methods such as the above site-directed mutagenesis. The suitable number is 1 to dozens, preferably 1 to 20, more preferably 1 to 10, further preferably 1 to 5.

The expression "one or more amino acid residues are deleted, substituted or added in any of the amino acid sequences shown in SEQ ID NOS: 19 to 25 and 68 and the amino acid sequences of a protein having proline iminopeptidase activity and a protein having L-amino acid amide hydrolase activity" means that the amino acid sequence may contain deletion, substitution or addition of a single or plural amino acid residues at an arbitrary position therein.

Deletion, substitution and addition may be simultaneously contained in one sequence, and amino acids to be substituted or added may be either natural or not. Examples of the natural amino acids are L-alanine, L-asparagine, L-aspartic acid, L-arginine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine and L-cysteine.

The following are examples of the amino acids capable of mutual substitution. The amino acids in the same group can be mutually substituted.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine

Group B: aspartic acid, glutamic acid, isoaspartic acid,

isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid

Group C: asparagine, glutamine

Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic
5 acid, 2,3-diaminopropionic acid

Group E: proline, 3-hydroxyproline, 4-hydroxyproline

Group F: serine, threonine, homoserine

Group G: phenylalanine, tyrosine

There is not any specific restriction as to the
10 position where the above deletion, substitution or
addition of one or more amino acid residues is introduced,
so long as a protein having an amino acid sequence
carrying the introduced mutation has the dipeptide-
synthesizing activity. Suitable examples include amino
15 acid residues which are not conserved in one or more amino
acid sequences among the amino acid sequences shown in SEQ
ID NOS: 19 to 25 and 68 when these sequences are compared.

The above proteins consisting of an amino acid
sequence wherein one or more amino acid residues are
20 deleted, substituted or added and having the activity to
synthesize a dipeptide include a protein consisting of an
amino acid sequence which has usually 65% or more,
preferably 80% or more, more preferably 90% or more,
particularly preferably 95% or more homology to the amino
25 acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68,
and a protein consisting of an amino acid sequence which
has usually 80% or more, preferably 90% or more, more
preferably 95% or more homology to the amino acid sequence
of proline iminopeptidase or L-amino acid amide hydrolase.

30 The homology among amino acid sequences and
nucleotide sequences can be determined by using programs
such as BLAST and FASTA described above.

The amino acid sequence shown in SEQ ID NO: 33 is a
region conserved among the proteins having the amino acid
35 sequences shown in SEQ ID NOS: 19 to 25 and is also a
region corresponding to the consensus sequence of proteins

having Ala-Ala ligase activity derived from various microorganisms.

Microorganisms producing a protein having an amino acid sequence which has 80% or more, preferably 90% or more, further preferably 95% or more homology to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide are also included in the dipeptide-producing microorganisms.

In order that the protein having an amino acid sequence which has 80% or more, preferably 90% or more, further preferably 95% or more homology to the amino acid sequence shown in SEQ ID NO: 33 may have the activity to synthesize a dipeptide, it is desirable that the homology of its amino acid sequence to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 is at least 80% or more, usually 90% or more, and particularly 95% or more.

The homology among amino acid sequences can be determined by using programs such as BLAST and FASTA described above.

The microorganisms of the present invention also include microorganisms carrying a recombinant DNA obtained by ligating, to a vector DNA, DNA encoding a protein having the activity to synthesize a dipeptide by condensation and ligation of one or more kinds of amino acids, DNA encoding a protein having the activity to synthesize a dipeptide from an L-amino acid ester and an L-amino acid, or DNA encoding a protein having the activity to synthesize a dipeptide from an L-amino acid amide and an L-amino acid.

Examples of the microorganisms include those belonging to the genera Escherichia, Bacillus, Corynebacterium and Saccharomyces.

The DNAs encoding a protein having the activity to synthesize a dipeptide by condensation and ligation of one or more kinds of amino acids include DNAs encoding NRPS, D-Ala-D-Ala ligase or bacilysin synthetase.

Examples of the DNAs encoding NRPS include DNAs encoding a protein selected from the group consisting of BacA, BacB, BacC, TycA, TycB, TycC and PcbAB.

Examples of the DNAs encoding D-Ala-D-Ala ligase
5 include DNAs encoding a protein selected from the group consisting of DdlA, DdlB and DdlC.

Examples of the DNAs encoding bacilysin synthetase include DNAs encoding proteins of the following [1] to [4]:

- 10 [1] a protein having the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68;
[2] a protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted or added in the amino acid sequence shown in any of SEQ ID
15 NOS: 19 to 25 and 68 and having the activity to synthesize a dipeptide;
[3] a protein consisting of an amino acid sequence which has 65% or more homology to the amino acid sequence shown in any of SEQ ID NOS: 19 to 25 and 68 and having the
20 activity to synthesize a dipeptide; and
[4] a protein having an amino acid sequence which has 80% or more homology to the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;
25 and DNAs of the following [5] to [7]:
[5] DNA having the nucleotide sequence shown in any of SEQ ID NOS: 26 to 32, 64 and 65;
[6] DNA which hybridizes with DNA having the nucleotide sequence shown in any of SEQ ID NOS: 26 to 32, 64 and 65
30 under stringent conditions and which encodes a protein having the activity to synthesize a dipeptide; and
[7] DNA having a nucleotide sequence which has 80% or more homology to the nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a
35 dipeptide.

Examples of the DNAs encoding a protein having the

activity to synthesize a dipeptide from an L-amino acid ester and an L-amino acid include DNAs encoding proteins of the following [1] to [3]:

- [1] proline iminopeptidase described in any of
5 WO03/010307; FEMS Microbiol. Lett., 78, 191 (1999);
Japanese Published Unexamined Patent Application No.
113887/90; Arch. Biochem. Biophys., 336, 35 (1996); J.
Biochem., 119, 468 (1996); Microbiology, 140, 527 (1994);
J. Bacteriol., 174, 7919 (1994); J. Biochem., 116, 818
10 (1994); Japanese Published Unexamined Patent Application
No. 121860/97; Mol. Microbiol., 9, 1203 (1993); Appl.
Environ. Microbiol., 64, 4736 (1998); J. Biochem., 122,
601 (1997); FEBS Lett., 398, 101 (1996); and Nature, 406,
959 (2000);
- 15 [2] a protein consisting of an amino acid sequence wherein
one or more amino acid residues are deleted, substituted
or added in the amino acid sequence of any proline
iminopeptidase of the above [1] and having proline
iminopeptidase activity; and
- 20 [3] a protein consisting of an amino acid sequence which
has 80% or more homology to the amino acid sequence of any
proline iminopeptidase of the above [1] and having proline
iminopeptidase activity;
and DNAs of the following [4] and [5]:
- 25 [4] DNA encoding proline iminopeptidase and having the
nucleotide sequence described in any of WO03/010307; FEMS
Microbiol. Lett., 78, 191 (1999); Japanese Published
Unexamined Patent Application No. 113887/90; Arch. Biochem.
Biophys., 336, 35 (1996); J. Biochem., 119, 468 (1996);
30 Microbiology, 140, 527 (1994); J. Bacteriol., 174, 7919
(1994); J. Biochem., 116, 818 (1994); Japanese Published
Unexamined Patent Application No. 121860/97; Mol.
Microbiol., 9, 1203 (1993); Appl. Environ. Microbiol., 64,
4736 (1998); J. Biochem., 122, 601 (1997); FEBS Lett., 398,
35 101 (1996); and Nature, 406, 959 (2000); and
[5] DNA which hybridizes with any DNA encoding proline

iminopeptidase of the above [4] under stringent conditions and which encodes a protein having proline iminopeptidase activity.

5 Examples of the DNAs encoding a protein having the activity to synthesize a dipeptide from an L-amino acid amide and an L-amino acid include DNAs encoding proteins of the following [1] and [2]:

[1] a protein consisting of an amino acid sequence wherein one or more amino acid residues are deleted, substituted
10 or added in the amino acid sequence of L-amino acid amide hydrolase described in WO03/010187 and having L-amino acid amide hydrolase activity; and

[2] a protein consisting of an amino acid sequence which has 80% or more homology to the amino acid sequence of L-
15 amino acid amide hydrolase described in WO03/010187 and having L-amino acid amide hydrolase activity;

and DNAs of the following [3] and [4]:

[3] DNA having the nucleotide sequence described in WO03/010187 and encoding L-amino acid amide hydrolase; and

20 [4] DNA which hybridizes with DNA consisting of the nucleotide sequence described in WO03/010187 and encoding L-amino acid amide hydrolase under stringent conditions and which encodes a protein having L-amino acid amide hydrolase activity.

25 The above DNA capable of hybridization under stringent conditions refers to DNA which is obtained by colony hybridization, plaque hybridization, Southern blot hybridization, or the like using a part or the whole of any of the above DNAs as a probe. A specific example of
30 such DNA is DNA which can be identified by performing hybridization at 65°C in the presence of 0.7 to 1.0 mol/l, preferably 0.9 mol/l sodium chloride using a filter with colony- or plaque-derived DNA immobilized thereon, and then washing the filter at 65°C with a 0.1 to 2-fold conc.,
35 preferably 0.1-fold conc. SSC solution (1-fold conc. SSC solution: 150 mmol/l sodium chloride and 15 mmol/l sodium

citrate). Hybridization can be carried out according to the methods described in Molecular Cloning, Second Edition; Current Protocols in Molecular Biology; DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995), etc. Specifically, the
5 hybridizable DNA includes DNA having at least 80% or more homology, preferably 90% or more homology, more preferably 95% or more homology to the nucleotide sequence of any of the above DNAs as calculated by use of a program such as
10 BLAST or FASTA described above based on the above parameters.

It is possible to confirm that the DNA hybridizing with the above DNA under stringent conditions is DNA encoding a protein having the activity to synthesize a
15 dipeptide in the following manner. That is, a recombinant DNA expressing the DNA is prepared and the recombinant DNA is introduced into a host cell to obtain a microorganism to be used as an enzyme source. Then, 1) the enzyme source and one or more kinds of amino acids are allowed to
20 be present in an aqueous medium, followed by HPLC analysis or the like to know whether a dipeptide is formed and accumulated in the aqueous medium, 2) the enzyme source, an L-amino acid ester and an L-amino acid are allowed to be present in an aqueous medium, followed by HPLC analysis
25 or the like to know whether a dipeptide is formed and accumulated in the aqueous medium, or 3) the enzyme source, an L-amino acid amide and an L-amino acid are allowed to be present in an aqueous medium, followed by HPLC analysis or the like to know whether a dipeptide is formed and
30 accumulated in the aqueous medium.

The homology among nucleotide sequences can be determined by using programs such as BLAST and FASTA described above.

35 2. Methods for Preparing the Microorganisms of the Present Invention

The microorganisms of the present invention can be obtained by any of the following methods: 1) methods of imparting the dipeptide-producing ability to microorganisms in which the functions of one or more kinds of peptidases and one or more kinds of proteins having peptide-transporting activity are reduced or lost, or microorganisms in which the functions of three or more kinds of peptidases are reduced or lost; and 2) methods of reducing or causing loss of the functions of a) one or more kinds of peptidases and one or more kinds of peptide-transporting proteins or b) three or more kinds of peptidases of microorganisms having the ability to produce a dipeptide.

- (1) Methods of imparting the dipeptide-producing ability to microorganisms in which the functions of one or more kinds of peptidases and one or more kinds of peptide-transporting proteins are reduced or lost, or microorganisms in which the functions of three or more kinds of peptidases are reduced or lost
- (i) Preparation of microorganisms in which the activities of peptidases and peptide-transporting proteins are reduced or lost

The microorganisms in which the activities of peptidases and peptide-transporting proteins are reduced or lost may be obtained by any method capable of preparing such microorganisms. For example, they can be obtained by introducing a deletion, substitution or addition of a nucleotide into peptidase genes and genes encoding peptide-transporting proteins on chromosomal DNAs of microorganisms as described below.

The methods for introducing a deletion, substitution or addition of a nucleotide into a gene on the chromosomal DNA of a microorganism include methods utilizing homologous recombination. An example of the methods utilizing general homologous recombination is a method

using a plasmid for homologous recombination prepared by ligating a mutant gene having an introduced nucleotide deletion, substitution or addition with a plasmid DNA incapable of autonomous replication in a host cell into
5 which the nucleotide deletion or the like is to be introduced and carrying a drug resistance gene.

The plasmid for homologous recombination is introduced into a host cell by an ordinary method, followed by selection of a transformant in which the
10 plasmid for homologous recombination has been integrated into the chromosomal DNA by homologous recombination using the drug resistance as a marker. The obtained transformant is cultured using a medium which does not contain the drug for several hours to one day, and then
15 spread on an agar medium containing the drug and on an agar medium without the drug. By selecting a strain which does not grow on the former medium but can grow on the latter medium, the strain in which second homologous recombination occurred on the chromosomal DNA can be
20 obtained. Introduction of a nucleotide deletion, substitution or addition into a desired gene on the chromosomal DNA can be confirmed by determining the nucleotide sequence of a region of the chromosomal DNA containing the gene into which the deletion or the like
25 has been introduced.

By use of the above method, a nucleotide deletion, substitution or addition can be introduced into desired genes on chromosomal DNAs of microorganisms such as those belonging to the genera Escherichia, Bacillus,
30 Corynebacterium and Saccharomyces.

Further, a nucleotide deletion, substitution or addition can be efficiently introduced into plural genes by utilizing homologous recombination according to a method using a linear DNA.

35 Specifically, a linear DNA containing a gene into which a nucleotide deletion, substitution or addition is

to be introduced is incorporated into a cell to cause homologous recombination between chromosomal DNA and the introduced linear DNA. This method is applicable to any microorganisms capable of efficiently incorporating a linear DNA. Preferred microorganisms are those belonging to the genera Escherichia and Bacillus. Escherichia coli is more preferred, and Escherichia coli expressing a group of recombinant proteins derived from λ phage (Red recombination system) is further preferred.

10 An example of Escherichia coli expressing λ Red recombination system is Escherichia coli JM101 carrying pKD46, which is a plasmid DNA comprising a λ Red recombination system gene (available from Escherichia coli Genetic Stock Center, Yale University, U.S.A.)

15 Examples of the DNAs useful for homologous recombination are as follows:

- (a) linear DNA in which DNAs present on the outside of a region of chromosomal DNA to be subjected to introduction of a nucleotide deletion, substitution or addition or DNAs having homology to the DNAs are present at both termini of a drug resistance gene;
- (b) linear DNA in which DNAs present on the outside of a region of chromosomal DNA to be subjected to introduction of a nucleotide deletion, substitution or addition or DNAs having homology to the DNAs are directly ligated with each other;
- (c) linear DNA in which DNAs present on the outside of a region of chromosomal DNA to be subjected to introduction of a nucleotide deletion, substitution or addition or DNAs having homology to the DNAs are present at both termini of a drug resistance gene and a gene that can be used for negative selection; and
- (d) linear DNA of the above (a) in which a nucleotide sequence recognized by yeast-derived Flp recombinase [Proc. Natl. Acad. Sci. USA., 82, 5875 (1985)] is additionally present between the drug resistance gene and DNAs present

on the outside of a region of chromosomal DNA or DNAs having homology to the DNAs.

As the drug resistance gene, any drug resistance genes that impart resistance to a drug to which the host microorganism shows sensitivity can be used. When Escherichia coli is used as the host microorganism, examples of the drug resistance genes are kanamycin resistance gene, chloramphenicol resistance gene, gentamicin resistance gene, spectinomycin resistance gene, tetracycline resistance gene and ampicillin resistance gene.

The "gene that can be used for negative selection" refers to a gene that is fatal to a host microorganism under certain culture conditions when the gene is expressed in the host microorganism. Examples of the genes are sacB gene derived from a microorganism belonging to the genus Bacillus [Appl. Environ. Microbiol., 59, 1361-1366 (1993)] and rpsL gene derived from a microorganism belonging to the genus Escherichia [Genomics, 72, 99-104 (2001)].

The DNAs present on the outside of a region of chromosomal DNA to be subjected to introduction of a substitution or deletion or DNAs having homology to the DNAs in the above linear DNAs are located in the same direction as that on the chromosomal DNA, and their length is preferably about 10 bp to 100 bp, more preferably about 20 bp to 50 bp, and further preferably about 30 bp to 40 bp.

The nucleotide sequence recognized by yeast-derived Flp recombinase is not specifically limited so long as it is a nucleotide sequence recognized by the said protein and catalyzing homologous recombination. Preferred examples are DNA having the nucleotide sequence shown in SEQ ID NO: 39, and DNA having a nucleotide sequence wherein one to several nucleotides are deleted, substituted or added in the said DNA and having a

nucleotide sequence recognized by yeast-derived Flp recombinase and catalyzing homologous recombination.

The "DNA having homology" refers to DNA having such a degree of identity that allows occurrence of homologous recombination between the subject region of chromosomal DNA and the above linear DNA, specifically, DNA having 80% or more homology, preferably 90% or more homology, more preferably 95% or more homology, further preferably 100% homology.

10 The homology among nucleotide sequences can be determined by using programs such as BLAST and FASTA described above.

The above linear DNA fragments can be prepared by PCR. The desired linear DNA can also be obtained by constructing DNA containing the above linear DNA on plasmid and then carrying out treatment with restriction enzymes.

Examples of the methods for introducing a nucleotide deletion, substitution or addition into the chromosomal DNA of a microorganism include the following Methods 1 to 4.

Method 1:

A method which comprises introducing the linear DNA of the above (a) or (d) into a host microorganism and selecting a transformant carrying the linear DNA inserted on its chromosomal DNA by homologous recombination using the drug resistance as a marker.

Method 2:

A method which comprises introducing the DNA, in which DNAs present on the outside of a region of chromosomal DNA to be subjected to introduction of a nucleotide deletion, substitution or addition or DNAs having homology to the DNAs are directly ligated with each other, into the transformant obtained according to the above Method 1 and eliminating the drug resistance gene inserted on its chromosomal DNA according to the method to

substitute or delete a region of the chromosomal DNA of the microorganism.

Method 3:

A method which comprises:

- 5 [1] introducing the linear DNA of the above (c) into a host microorganism and selecting a transformant carrying the linear DNA inserted on its chromosomal DNA by homologous recombination using the drug resistance as a marker;
- 10 [2] synthesizing DNA by ligating DNAs having homology to the DNAs present on the outside of a region of chromosomal DNA to be subjected to introduction of a substitution or deletion in the same direction as that on the chromosomal DNA, and introducing the synthesized DNA into the
- 15 transformant obtained in the above [1]; and
- [3] culturing the transformant subjected to the operation of the above [2] under conditions such that the gene that can be used for negative selection is expressed, and selecting a strain capable of growing by the culturing as
- 20 a strain in which the drug resistance gene and the gene that can be used for negative selection are eliminated from the chromosomal DNA.

Method 4:

A method which comprises:

- 25 [1] introducing the linear DNA of the above (d) into a host microorganism and selecting a transformant carrying the linear DNA inserted on its chromosomal DNA by homologous recombination using the drug resistance as a marker; and
- 30 [2] introducing a Flp recombinase gene expression plasmid into the transformant obtained in the above [1], and after expression of the gene, obtaining a strain sensitive to the drug used in the above [1].

In the above methods, introduction of the linear DNA
35 into a host microorganism can be carried out by any of the methods for introducing DNA into the microorganism, for

example, the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 248394/88) and electroporation [Nucleic Acids Res., 16,
5 6127 (1988)].

By using a DNA in which an arbitrary gene to be inserted to chromosomal DNA is incorporated in the center part of the DNA used in Method 2 or Method 3 [2], it is possible to eliminate the drug resistance gene and at the
10 same time to insert an arbitrary gene to the chromosomal DNA.

The above Methods 2 to 4 are methods that leave no foreign genes such as a drug resistance gene and a gene usable for negative selection on the chromosomal DNA of
15 the transformant to be finally obtained. Therefore, it is possible to readily produce a microorganism having nucleotide deletions, substitutions or additions in two or more different regions of the chromosomal DNA by repeating the operations of Method 2, Method 3 [1] to [3], and
20 Method 4 [1] and [2] using the same drug resistance gene and the same gene usable for negative selection.

(ii) Methods of imparting the dipeptide-producing ability

The methods of imparting the dipeptide-producing
25 ability to the microorganisms used in the above (i) include the following methods.

(a) Preparation of DNA encoding a protein having the activity to synthesize a dipeptide

30 The above DNA encoding a protein having the activity to synthesize a dipeptide can be obtained by the methods described below utilizing nucleotide sequence information on the DNA.

For example, the DNA encoding bacilysin synthetase
35 can be obtained by Southern hybridization of a chromosomal DNA library from a microorganism belonging to the genus

Bacillus using a probe designed based on the nucleotide sequence shown in any of SEQ ID NOS: 26 to 32, 64 and 65, or by PCR [PCR Protocols, Academic Press (1990)] using primer DNAs designed based on the nucleotide sequence
5 shown in any of SEQ ID NOS: 26 to 32, 64 and 65 and, as a template, the chromosomal DNA of a microorganism belonging to the genus Bacillus.

The DNA encoding a protein having the activity to synthesize a dipeptide can also be obtained by conducting
10 a search through various gene sequence databases for a sequence having 65% or more homology, preferably 80% or more homology, more preferably 90% or more homology, further preferably 95% or more homology to the nucleotide sequence of DNA encoding the amino acid sequence shown in
15 any of SEQ ID NOS: 19 to 25, 33 and 68, and obtaining the desired DNA, based on the nucleotide sequence obtained by the search, from a chromosomal DNA or cDNA library of an organism having the nucleotide sequence according to the above-described method.

20 The obtained DNA, as such or after cleavage with appropriate restriction enzymes, is inserted into a vector by a conventional method to obtain a recombinant DNA. A plasmid DNA is extracted from a transformant obtained by introducing the recombinant DNA into Escherichia coli.
25 Then, the nucleotide sequence of the DNA can be determined by a conventional sequencing method such as the dideoxy method [Proc. Natl. Acad. Sci., USA, 74, 5463 (1977)] or by using a nucleotide sequencer such as 373A DNA Sequencer (Perkin-Elmer Corp.).

30 In cases where the obtained DNA is found to be a partial DNA by the analysis of nucleotide sequence, the full length DNA can be obtained by Southern hybridization of a chromosomal DNA library using the partial DNA as a probe.

35 It is also possible to prepare the desired DNA by chemical synthesis using a DNA synthesizer (e.g., Model

8905, PerSeptive Biosystems) based on the determined nucleotide sequence of the DNA.

Examples of the DNAs that can be obtained by the above-described method are DNAs having the nucleotide sequences shown in SEQ ID NOS: 26 to 32, 64 and 65.

Examples of the vectors for inserting the DNA include pBluescriptII KS(+) (Stratagene), pDIRECT [Nucleic Acids Res., 18, 6069 (1990)], pCR-Script Amp SK(+) (Stratagene), pT7 Blue (Novagen, Inc.), pCR II (Invitrogen Corp.) and pCR-TRAP (Genhunter Corp.).

Examples of Escherichia coli include Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM101, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Escherichia coli MP347, Escherichia coli NM522 and Escherichia coli ME8415.

Introduction of the recombinant DNA can be carried out by any of the methods for introducing DNA into the above host cells, for example, the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 248394/88) and electroporation [Nucleic Acids Res., 16, 6127 (1988)].

An example of the microorganism carrying the DNA encoding a protein having the dipeptide-synthesizing activity obtained by the above method is Escherichia coli NM522/pPE43, which is a microorganism carrying a recombinant DNA comprising DNA having the sequence shown in SEQ ID NO: 19 described below.

(b) Production of a protein having the dipeptide-synthesizing activity

The protein having the dipeptide-synthesizing activity can be produced by expressing the DNA obtained by

the methods described in the above (a) in host cells using the methods described in Molecular Cloning, Second Edition, Current Protocols in Molecular Biology, etc., for example, in the following manner.

5 On the basis of the DNA obtained by the methods described in the above (a), a DNA fragment of an appropriate length comprising a region encoding the protein having the dipeptide synthesizing-activity is prepared according to need. The productivity of the
10 protein can be enhanced by replacing a nucleotide in the nucleotide sequence of the region encoding the protein so as to make a codon most suitable for the expression in a host cell.

 The DNA fragment is inserted downstream of a
15 promoter in an appropriate expression vector to prepare a recombinant DNA.

 A transformant producing the protein having the dipeptide synthesizing-activity can be obtained by introducing the recombinant DNA into a host cell suited
20 for the expression vector.

 As the host cell, any microorganisms such as bacterial cells and yeast cells that are capable of expressing the desired gene can be used.

 The expression vectors that can be employed are
25 those capable of autonomous replication or integration into the chromosome in the above host cells and comprising a promoter at a position appropriate for the transcription of the DNA encoding the protein having the dipeptide synthesizing-activity.

30 When a procaryote such as a bacterium is used as the host cell, it is preferred that the recombinant DNA comprising the DNA encoding a protein having the dipeptide-synthesizing activity is a recombinant DNA which is capable of autonomous replication in the procaryote and
35 which comprises a promoter, a ribosome binding sequence, the DNA encoding a protein having the dipeptide-

synthesizing activity, and a transcription termination sequence. The recombinant DNA may further comprise a gene regulating the promoter.

Examples of suitable expression vectors are pBTrp2, 5 pBTac1 and pBTac2 (products of Boehringer Mannheim GmbH), pHelix1 (Roche Diagnostics Corp.), pKK233-2 (Amersham Pharmacia Biotech), pSE280 (Invitrogen Corp.), pGEMEX-1 (Promega Corp.), pQE-8 (Qiagen, Inc.), pET-3 (Novagen, Inc.), pKYP10 (Japanese Published Unexamined Patent 10 Application No. 110600/83), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II SK(+), pBluescript II KS(-) (Stratagene), pTrS30 [prepared from Escherichia coli 15 JM109/pTrS30 (FERM BP-5407)], pTrS32 [prepared from Escherichia coli JM109/pTrS32 (FERM BP-5408)], pPAC31 (WO98/12343), pUC19 [Gene, 33, 103 (1985)], pSTV28 (Takara Shuzo Co., Ltd.), pUC118 (Takara Shuzo Co., Ltd.) and pPA1 (Japanese Published Unexamined Patent Application No. 20 233798/88).

As the promoter, any promoters capable of functioning in host cells such as Escherichia coli can be used. For example, promoters derived from Escherichia coli or phage, such as trp promoter (P_{trp}), lac promoter 25 (P_{lac}), P_L promoter, P_R promoter and P_{SE} promoter, SP01 promoter, SP02 promoter and penP promoter can be used. Artificially designed and modified promoters such as a promoter in which two P_{trp} s are combined in tandem, tac promoter, lacT7 promoter and letI promoter, etc. can also 30 be used.

Also useful are promoters such as xylA promoter for the expression in bacteria belonging to the genus Bacillus [Appl. Microbiol. Biotechnol., 35, 594-599 (1991)] and P54-6 promoter for the expression in bacteria belonging to 35 the genus Corynebacterium [Appl. Microbiol. Biotechnol., 53, 674-679 (2000)].

It is preferred to use a plasmid in which the distance between the Shine-Dalgarno sequence (ribosome binding sequence) and the initiation codon is adjusted to an appropriate length (e.g., 6 to 18 nucleotides).

5 In the recombinant DNA wherein the DNA encoding the protein having the dipeptide synthesizing-activity is ligated to an expression vector, the transcription termination sequence is not essential, but it is preferred to place the transcription termination sequence
10 immediately downstream of the structural gene.

An example of such recombinant DNA is pPE43 described below.

Examples of procaryotes suitable for use as host cells include microorganisms belonging to the genera
15 Escherichia, Bacillus and Corynebacterium. Specific examples are Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli DH5 α , Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM101,
20 Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Escherichia coli MP347, Escherichia coli NM522, Bacillus subtilis ATCC 33712, Bacillus megaterium, Bacillus sp. FERM BP-6030, Bacillus
25 amyloliquefaciens, Bacillus coagulans, Bacillus licheniformis, Bacillus pumilus, Corynebacterium glutamicum ATCC 13032 and Corynebacterium glutamicum ATCC 14297.

Introduction of the recombinant DNA can be carried
30 out by any of the methods for introducing DNA into the above host cells, for example, the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 248394/88) and electroporation [Nucleic
35 Acids Res., 16, 6127 (1988)].

When a strain belonging to the genus Saccharomyces

is used as the host cell, YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19, pHS15, etc. can be used as the expression vector.

As the promoter, any promoters capable of functioning in strains belonging to the genus Saccharomyces can be used. Suitable promoters include PH05 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock polypeptide promoter, MF α 1 promoter and CUP 1 promoter.

Examples of suitable host cells are strains belonging to the genus Saccharomyces, specifically, Saccharomyces cerevisiae.

Introduction of the recombinant DNA can be carried out by any of the methods for introducing DNA into yeast, for example, electroporation [Methods Enzymol., 194, 182 (1990)], the spheroplast method [Proc. Natl. Acad. Sci. USA, 81, 4889 (1984)] and the lithium acetate method [J. Bacteriol., 153, 163 (1983)].

(2) Methods of reducing or causing loss of the functions of a) one or more kinds of peptidases and one or more kinds of peptide-transporting proteins or b) three or more kinds of peptidases of microorganisms having the ability to produce a dipeptide

Microorganisms having the ability to produce a dipeptide can be prepared by carrying out the methods described in the above (1) (ii) using arbitrary microorganisms as host cells. By carrying out the methods described in the above (1) (i) using the microorganisms thus prepared, microorganisms in which the functions of one or more kinds of peptidases and one or more kinds of peptide-transporting proteins or the functions of three or more kinds of peptidases are reduced or lost and which have the ability to produce a dipeptide can be prepared.

The microorganisms of the present invention can also be obtained by carrying out the methods described in the

above (1) (i) using microorganisms inherently having the ability to produce a dipeptide.

Examples of the above microorganisms include those belonging to the genera Escherichia, Bacillus,
5 Corynebacterium and Saccharomyces. Preferred are Escherichia coli, Bacillus subtilis, Corynebacterium glutamicum and Saccharomyces cerevisiae.

10 3. Process for Producing a Dipeptide of the Present Invention

The production processes of the present invention include:

- 15 (i) a process for producing a dipeptide, which comprises: allowing an enzyme source and one or more kinds of amino acids to be present in an aqueous medium, said enzyme source being a culture of the microorganism of the present invention or a treated matter of the culture; allowing the dipeptide to form and accumulate in the aqueous medium; and recovering the dipeptide from the medium;
- 20 (ii) a process for producing a dipeptide, which comprises: allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being a culture of the microorganism of the present invention or a treated matter of the culture; allowing the dipeptide to form and accumulate in the aqueous medium; and recovering the dipeptide from the medium; and
- 25 (iii) a process for producing a dipeptide, which comprises: allowing an enzyme source, an L-amino acid amide and an L-amino acid to be present in an aqueous medium, said enzyme source being a culture of the microorganism of the present invention or a treated matter of the culture; allowing the dipeptide to form and accumulate in the aqueous medium; and recovering the dipeptide from the medium.

35 One or more kinds, preferably one or two kinds of amino acids used as substrates in the above production

process (i) are amino acids, preferably amino acid selected from the group consisting of L-amino acids, glycine (Gly) and β -alanine (β -Ala), and can be used in any combination. Examples of L-amino acids are L-alanine (L-Ala), L-glutamine (L-Gln), L-glutamic acid (L-Glu), L-valine (L-Val), L-leucine (L-Leu), L-isoleucine (L-Ile), L-proline (L-Pro), L-phenylalanine (L-Phe), L-tryptophan (L-Trp), L-methionine (L-Met), L-serine (L-Ser), L-threonine (L-Thr), L-cysteine (L-Cys), L-asparagine (L-Asn), L-tyrosine (L-Tyr), L-lysine (L-Lys), L-arginine (L-Arg), L-histidine (L-His), L-aspartic acid (L-Asp), L- α -aminobutyric acid (L- α -AB), L-azaserine, L-theanine, L-4-hydroxyproline (L-4-HYP), L-3-hydroxyproline (L-3-HYP), L-ornithine (L-Orn), L-citrulline (L-Cit) and L-6-diazo-5-oxo-norleucine.

The amino acids which are more preferably used in the above process (i) include the following: a combination of one kind of amino acid selected from the group consisting of L-Ala, Gly, L-Met, L-Ser, L-Thr and β -Ala, and one kind of amino acid selected from the group consisting of L-Ala, L-Gln, L-Glu, Gly, L-Val, L-Leu, L-Ile, L-Pro, L-Phe, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Asn, L-Tyr, L-Lys, L-Arg, L-His, L-Asp, L- α -AB, β -Ala, L-azaserine, L-theanine, L-4-HYP, L-3-HYP, L-Orn, L-Cit and L-6-diazo-5-oxo-norleucine; a combination of L-Gln and L-Phe; and a combination of L- α -AB and L-Gln, L-Arg or L- α -AB. Further preferred amino acids are: a combination of L-Ala and one kind of amino acid selected from the group consisting of L-Ala, L-Gln, Gly, L-Val, L-Leu, L-Ile, L-Phe, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Asn, L-Tyr, L-Lys, L-Arg, L-His, L- α -AB, L-azaserine, L-Cit and L-theanine; a combination of Gly and one kind of amino acid selected from the group consisting of L-Gln, Gly, L-Phe, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Tyr, L-Lys, L-Arg, L- α -AB and L-Cit; a combination of L-Met and one kind of amino acid selected from the group consisting of L-Phe, L-

Met, L-Ser, L-Thr, L-Cys, L-Tyr, L-Lys and L-His; a combination of L-Ser and one kind of amino acid selected from the group consisting of L-Gln, L-Phe, L-Ser, L-Thr, L-Tyr, L-His and L- α -AB; a combination of L-Thr and one
 5 kind of amino acid selected from the group consisting of L-Gln, L-Phe, L-Leu, L-Thr and L- α -AB; a combination of L-Gln and L-Phe; a combination of β -Ala and one kind of amino acid selected from the group consisting of L-Phe, L-Met, L-His and L-Cit; and a combination of L- α -AB and L-
 10 Gln, L-Arg or L- α -AB.

In the above production process (i), the L-amino acid used as a substrate is added to the aqueous medium at the start or in the course of reaction to give a concentration of 0.1 to 500 g/l, preferably 0.2 to 200 g/l.

15 The dipeptides produced by the above process (i) include the dipeptides represented by the following formula (I):



20

(wherein R^1 and R^2 , which may be the same or different, each represent an amino acid). Preferred dipeptides are those represented by the above formula (I) wherein R^1 and R^2 , which may be the same or different, each represent an
 25 amino acid selected from the group consisting of L-Ala, L-Gln, L-Glu, Gly, L-Val, L-Leu, L-Ile, L-Pro, L-Phe, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Asn, L-Tyr, L-Lys, L-Arg, L-His, L-Asp, L- α -AB, β -Ala, L-azaserine, L-theanine, L-4-HYP, L-3-HYP, L-Orn and L-6-diazo-5-oxo-norleucine. More
 30 preferred are dipeptides wherein R^1 is L-Ala, Gly, L-Met, L-Ser, L-Thr or β -Ala, and R^2 is L-Gln, L-Glu, Gly, L-Val, L-Leu, L-Ile, L-Pro, L-Phe, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Asn, L-Tyr, L-Lys, L-Arg, L-His, L-Asp, L- α -AB, β -Ala, L-azaserine, L-theanine, L-4-HYP, L-3-HYP, L-Orn or
 35 L-6-diazo-5-oxo-norleucine. Further preferred dipeptides are: dipeptides wherein R^1 is L-Ala and R^2 is L-Gln, Gly,

L-Val, L-Leu, L-Ile, L-Phe, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Asn, L-Tyr, L-Lys, L-Arg, L-His, L- α -AB, L-azaserine or L-theanine; dipeptides wherein R¹ is Gly and R² is L-Gln, Gly, L-Trp, L-Met, L-Ser, L-Thr, L-Cys, L-Tyr, L-Lys, L-Arg or L- α -AB; dipeptides wherein R¹ is L-Met and R² is L-Phe, L-Met, L-Cys, L-Tyr, L-Lys or L-His; dipeptides wherein R¹ is L-Ser and R² is L-Gln, Gly, L-Phe, L-Met, L-Ser, L-Thr, L-Tyr, L-His or L- α -AB; dipeptides wherein R¹ is L-Thr and R² is L-Gln, L-Gly, L-Phe, L-Met, L-Ser, L-Thr or L- α -AB; dipeptides wherein R¹ is L-Gln and R² is L-Phe or L- α -AB; a dipeptide wherein R¹ is L-Phe and R² is L-Gln; a dipeptide wherein R¹ is L-Trp and R² is Gly; dipeptides wherein R¹ is L-Cys and R² is L-Ala, L-Gln, Gly or L-Met; dipeptides wherein R¹ is L-Lys and R² is L-Ala, Gly or L-Met; a dipeptide wherein R¹ is L-Arg and R² is L- α -AB; a dipeptide wherein R¹ is L-His and R² is L-Met; and dipeptides wherein R¹ is L- α -AB and R² is L-Ala, L-Gln, Gly, L-Ser, L-Thr, L-Arg or L- α -AB.

Further, in the above process, compounds which can be metabolized by the microorganism of the present invention to produce ATP, for example, sugars such as glucose, alcohols such as ethanol, and organic acids such as acetic acid may be added, as ATP source, to the aqueous medium according to need.

The L-amino acid ester and L-amino acid used as substrates in the above production process (ii) may be any of L-amino acid esters and L-amino acids that can be used as substrates by the microorganism of the present invention to form a dipeptide, and they can be used in any combination. Preferably, the L-amino acid ester is selected from the group consisting of L-alanine ester, glycine ester, L-valine ester, L-isoleucine ester, L-methionine ester, L-phenylalanine ester, L-serine ester, L-threonine ester, L-glutamine ester, L-tyrosine ester, L-arginine ester, L-aspartic acid- α -ester, L-aspartic acid- β -ester, L-leucine ester, L-asparagine ester, L-lysine

ester, L-aspartic acid- α,β -dimethyl ester and L-glutamine- γ -ester, and the L-amino acid is selected from the group consisting of L-Gln, L-Asn, Gly, L-Ala, L-Leu, L-Met, L-Pro, L-Phe, L-Trp, L-Ser, L-Thr, L-Tyr, L-Lys, L-Arg, L-His and L-Glu.

In the above process (ii), the L-amino acid ester and L-amino acid used as substrates are added to the aqueous medium at the start or in the course of reaction to give a concentration of 0.1 to 500 g/l, preferably 0.2 to 200 g/l.

The L-amino acid amide and L-amino acid used as substrates in the above production process (iii) may be any of L-amino acid amides and L-amino acids that can be used as substrates by the microorganism of the present invention to form a dipeptide, and they can be used in any combination. Preferably, the L-amino acid amide is selected from the group consisting of L-alanine amide, glycine amide and L-aspartic acid amide, and the L-amino acid is selected from the group consisting of L-Gln, L-Asn, Gly, L-Ala, L-Val, L-Leu, L-Ile, L-Met, L-Pro, L-Phe, L-Trp, L-Ser, L-Thr, L-Tyr, L-Lys, L-Arg, L-His and L-Glu.

In the above process (iii), the L-amino acid amide and L-amino acid used as substrates are added to the aqueous medium at the start or in the course of reaction to give a concentration of 0.1 to 500 g/l, preferably 0.2 to 200 g/l.

The aqueous medium used in the production processes of the present invention may comprise any components and may have any composition so far as the dipeptide-forming reaction is not inhibited. Suitable aqueous media include water and buffers such as phosphate buffer, carbonate buffer, acetate buffer, borate buffer, citrate buffer and Tris buffer. The aqueous medium may comprise alcohols such as methanol and ethanol, esters such as ethyl acetate, ketones such as acetone, and amides such as acetamide.

The dipeptide-forming reaction is carried out in the

aqueous medium at pH 5 to 11, preferably pH 6 to 10, at 20 to 60°C, preferably 25 to 45°C, for 2 to 150 hours, preferably 6 to 120 hours.

If necessary, a surfactant or an organic solvent may further be added to the aqueous medium.

Any surfactant that promotes the formation of a dipeptide can be used. Suitable surfactants include nonionic surfactants such as polyoxyethylene octadecylamine (e.g., Nymeen S-215, NOF Corporation), cationic surfactants such as cetyltrimethylammonium bromide and alkyldimethylbenzylammonium chloride (e.g., Cation F2-40E, NOF Corporation), anionic surfactants such as lauroyl sarcosinate, and tertiary amines such as alkyldimethylamine (e.g., Tertiary Amine FB, NOF Corporation), which may be used alone or in combination. The surfactant is usually used at a concentration of 0.1 to 50 g/l. As the organic solvent, xylene, toluene, aliphatic alcohols, acetone, ethyl acetate, etc. may be used usually at a concentration of 0.1 to 50 ml/l.

The treated matters of the culture include concentrated culture, dried culture, cells obtained by centrifuging the culture, and products obtained by treating the cells by various means such as drying, freeze-drying, treatment with a surfactant, ultrasonication, mechanical friction, treatment with a solvent, enzymatic treatment and immobilization. The treated matters of the culture of the present invention also include crude extracts of protein obtained by removing insoluble matters and the like from the treated matters obtained by treating the above cells by means such as treatment with a surfactant, ultrasonication, mechanical friction, treatment with a solvent and enzymatic treatment.

When the culture or a treated matter of the culture is used as the enzyme source, the amount of the enzyme source to be added varies according to its specific

activity, etc., but is, for example, 5 to 1000 mg, preferably 10 to 400 mg per mg of amino acid, L-amino acid ester or L-amino acid amide used as a substrate.

Recovery of the dipeptide formed and accumulated in the aqueous medium can be carried out by ordinary methods using active carbon, ion-exchange resins, etc. or by means such as extraction with an organic solvent, crystallization, thin layer chromatography and high performance liquid chromatography.

Further, the above production processes (ii) and (iii) can be carried out according to the descriptions in WO03/010189 or WO03/010187.

Experimental examples of the present invention are shown below.

15

Experimental Example 1

Construction of a Plasmid Expressing ywfE Gene Derived from Bacillus subtilis

A ywfE gene fragment of Bacillus subtilis was obtained in the following manner.

By using a DNA synthesizer (Model 8905, PerSeptive Biosystems, Inc.), DNAs having the nucleotide sequences shown in SEQ ID NOS: 35 and 36 (hereinafter referred to as primer A and primer B, respectively) were synthesized. Primer A has a nucleotide sequence containing a region wherein the initiation codon of ywfE gene (atg) is substituted by the NcoI recognition sequence (ccatgg). Primer B has a nucleotide sequence containing a region wherein the termination codon of ywfE gene is substituted by the BamHI recognition sequence (ggatcc).

PCR was carried out using the chromosomal DNA of Bacillus subtilis as a template and the above primer A and primer B as a set of primers. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for one minute, reaction at 55°C for 2 minutes and reaction at 72°C for 3 minutes, using 40 μ l of a reaction

mixture comprising 0.1 μ g of the chromosomal DNA, 0.5 μ mol/l each of the primers, 2.5 units of Pfu DNA polymerase, 4 μ l of buffer for Pfu DNA polymerase (10 x) and 200 μ mol/l each of dNTPs.

5 One-tenth of the resulting reaction mixture was subjected to agarose gel electrophoresis to confirm that a ca. 1.4 kb fragment corresponding to the ywfE gene fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of
10 phenol/chloroform saturated with TE. The resulting mixture was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80°C for 30 minutes. The resulting solution was centrifuged, and the obtained DNA precipitate was
15 dissolved in 20 μ l of TE.

The thus obtained solution (5 μ l) was subjected to reaction to cleave the amplified DNA with restriction enzymes NcoI and BamHI. DNA fragments were separated by agarose gel electrophoresis, and a 1.4 kb DNA fragment
20 containing ywfE gene was recovered using GENECLAN II Kit (Bio 101).

C-Terminal His-tagged recombinant expression vector pQE60 (Qiagen, Inc.) (0.2 μ g) was cleaved with restriction enzymes NcoI and BamHI. DNA fragments were
25 separated by agarose gel electrophoresis, and a 3.4 kb DNA fragment was recovered in the same manner as above.

The 1.4 kb DNA fragment containing ywfE gene and the 3.4 kb DNA fragment obtained above were subjected to ligation reaction using a ligation kit (Takara Shuzo Co.,
30 Ltd.) at 16°C for 16 hours.

Escherichia coli NM522 (Stratagene) was transformed using the ligation reaction mixture according to the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], spread on LB agar medium containing 50 μ
35 g/ml ampicillin, and cultured overnight at 30°C.

A plasmid was extracted from a colony of the

transformant that grew on the medium according to a known method, whereby pQE60ywfe, which is a C-terminal His-tagged ywfe gene expression vector, was obtained. The structure of the vector was confirmed by digestion with
5 restriction enzymes (Fig. 1).

Experimental Example 2

Acquisition of a ywfe Gene Product

Escherichia coli NM522/pQE60ywfe carrying pQE60ywfe
10 was inoculated into 8 ml of LB medium containing 50 μ g/ml ampicillin in a test tube, and cultured at 28°C for 17 hours. The resulting culture was inoculated into 50 ml of LB medium containing 50 μ g/ml ampicillin in a 250-ml Erlenmeyer flask, and cultured at 30°C for 3 hours. Then,
15 isopropyl- β -D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mmol/l, followed by further culturing at 30°C for 4 hours. The resulting culture was centrifuged to obtain wet cells, and a His-tagged recombinant enzyme was purified from the wet cells
20 using HisTrap (His-tagged protein purification kit, Amersham Pharmacia Biotech) according to the instructions attached thereto.

Experimental Example 3

25 Production of Dipeptides Using the His-Tagged Recombinant Enzyme (1)

(i) A reaction mixture (0.1 ml) comprising 0.04 mg of the purified His-tagged recombinant enzyme obtained in Experimental Example 2, 100 mmol/l Tris-HCl (pH 8.0), 60
30 mmol/l magnesium chloride, 60 mmol/l ATP, 30 mmol/l L-Ala and 30 mmol/l L-Gln was prepared, and reaction was carried out at 37°C for 16 hours.

After the completion of reaction, the reaction product was derivatized by the dinitrophenol method and
35 then analyzed by HPLC. The HPLC analysis was carried out using, as a separation column, Lichrosorb-RP-18 column

(Kanto Kagaku) and, as an eluting solution, 1% (v/v) phosphoric acid and 25% (v/v) acetonitrile at a flow rate of 0.7 ml/min. As a result, it was confirmed that 3.7 g/l L-Ala-L-Gln and 0.3 g/l L-alanyl-L-alanine (L-Ala-L-Ala) were formed and accumulated in the reaction mixture.

(ii) Reactions were carried out under the same conditions as in the above (i) using reaction mixtures having the same composition as that of the reaction mixture of the above (i) except that 0.01 mg of the enzyme was used and L-Phe, L-Met, L-Leu and L-Val, respectively, were used in place of L-Gln.

After the completion of reactions, the reaction products were analyzed in the same manner as in the above (i), whereby it was confirmed that the following dipeptides were formed and accumulated in the respective reaction mixtures: 7.0 g/l L-alanyl-L-phenylalanine (L-Ala-L-Phe) alone; 7.0 g/l L-alanyl-L-methionine (L-Ala-L-Met) and 0.03 g/l L-Ala-L-Ala; 5.0 g/l L-alanyl-L-leucine (L-Ala-L-Leu) and 0.2 g/l L-Ala-L-Ala; and 1.6 g/l L-alanyl-L-valine (L-Ala-L-Val) and 0.3 g/l L-Ala-L-Ala.

(iii) Reactions were carried out under the same conditions as in the above (i) using reaction mixtures having the same composition as that of the reaction mixture of the above (i) except that 0.01 mg of the enzyme was used, Gly was used in place of L-Ala, and L-Phe and L-Met, respectively, were used in place of L-Gln.

After the completion of reactions, the reaction products were analyzed in the same manner as in the above (i), whereby it was confirmed that 5.2 g/l glycyl-L-phenylalanine (Gly-L-Phe) and 1.1 g/l glycyl-L-methionine (Gly-L-Met) were formed and accumulated in the respective reaction mixtures.

When ATP was excluded from the compositions of the above reaction mixtures, no dipeptide was formed.

The above results revealed that the ywfE gene product has the activity to produce, in the presence of ATP, the following dipeptides: L-Ala-L-Gln plus L-Ala-L-Ala, L-Ala-L-Phe, L-Ala-L-Met plus L-Ala-L-Ala, L-Ala-L-Leu plus L-Ala-L-Ala, or L-Ala-L-Val plus L-Ala-L-Ala from L-Ala plus L-Gln, L-Phe, L-Met, L-Leu or L-Val; and Gly-L-Phe or Gly-L-Met from Gly plus L-Phe or L-Met.

Experimental Example 4

10 Production of Dipeptides Using the His-Tagged Recombinant Enzyme (2)

A reaction mixture (0.1 ml) comprising 0.04 mg of the purified His-tagged recombinant enzyme obtained in Experimental Example 2, 100 mmol/l Tris-HCl (pH 8.0), 60
15 mmol/l magnesium chloride and 60 mmol/l ATP was prepared. To this mixture were respectively added combinations of various L-amino acids, Gly and β -Ala selected from the amino acids shown in the first row of Table 1 and in the leftmost column of Table 1 to give a concentration of 30
20 mmol/l each, and the resulting mixtures were subjected to reaction at 37°C for 16 hours. After the completion of reactions, the reaction products were analyzed by HPLC, whereby it was confirmed that the dipeptides shown in Table 1 were formed.

Table 1-1

	Ala	Gln	Glu	Gly	Val	Leu	Ile	Pro
Ala	AlaAla	AlaGln AlaAla	AlaAla	AlaGly AlaAla	AlaVal AlaAla	AlaLeu AlaAla	AlaIle AlaAla	AlaAla
Gln		X	X	GlyGln GlyGly	X	X	X	X
Glu				GlyGly				
Gly				GlyGly				GlyGly
Val								
Leu								
Ile								
Pro								
Phe								
Trp								
Met								
Ser								
Thr								
Cys								
Asn								
Tyr								
Lys								
Arg								
His								
Asp								
α AB								
β -Ala								
Cit								

Table 1-2

	Phe	Trp	Met	Ser	Thr	Cys	Asn	Tyr
Ala	AlaPhe AlaAla	AlaTrp AlaAla	AlaMet	AlaSer AlaAla	AlaThr AlaAla	AlaAla ○	AlaAsn AlaAla	AlaTyr AlaAla
Gln	○	×	MetMet	SerGln SerSer	ThrGln ThrThr	○	×	×
Glu								
Gly	GlyPhe	GlyGly ○	GlyMet GlyGly	GlySer GlyGly SerGly SerSer	GlyThr GlyGly ThrGly ThrThr	GlyGly ○	GlyGly	GlyTyr GlyGly
Val			×					
Leu			MetMet		ThrLeu			
Ile			MetMet					
Pro			MetMet	SerSer	ThrThr			
Phe			MetPhe MetMet	SerPhe	ThrPhe ThrThr			
Trp								
Met			MetMet	SerMet	ThrMet ThrThr	MetMet ○		MetTyr MetMet
Ser				SerSer	SerThr SerSer ThrSer ThrThr			SerTyr SerSer
Thr					ThrThr			
Cys								
Asn								
Tyr								
Lys								
Arg								
His								
Asp								
α -AB								
β -Ala								
Cit								

Table 1-3

	Lys	Arg	His	Asp	α -AB	β -Ala	Cit	Aza-serine	Theanine
Ala	AlaAla ○	AlaArg AlaAla	AlaHis AlaAla	AlaAla	AlaAla ○		AlaAla ○	AlaAla ○	AlaAla ○
Gln	×	×	×	×	○				
Glu									
Gly	GlyGly ○	GlyArg GlyGly	GlyGly	GlyGly	GlyGly ○		○		
Val									
Leu									
Ile									
Pro									
Phe				×		○			
Trp									
Met	MetMet ○		MetMet ○			○			
Ser			SerHis		SerSer ○				
Thr					ThrThr ○				
Cys									
Asn									
Tyr									
Lys									
Arg					○				
His						β -AlaHis			
Asp									
α -AB					○				
β -Ala									
Cit						○			

The dipeptides formed by the reaction using, as
 5 substrates, two (or one) kinds of L-amino acids, Gly and
 β -Ala shown in the first row and the leftmost column of
 Table 1 are shown in the respective cells of the table.
 In the table, ○ means that a dipeptide was formed though
 its sequence was unidentified; × means that formation of
 10 a dipeptide was not confirmed; and a blank means that
 reaction was not carried out.

Experimental Example 5

Production of a Dipeptide Using the Strain Expressing the His-Tagged Recombinant Enzyme

Escherichia coli NM522/pQE60ywfe obtained in
5 Experimental Example 1 was inoculated into 8 ml of LB medium containing 50 μ g/ml ampicillin in a test tube, and cultured at 28°C for 17 hours. The resulting culture was inoculated into 50 ml of LB medium containing 50 μ g/ml ampicillin in a 250-ml Erlenmeyer flask, and cultured at
10 30°C for 3 hours. Then, IPTG was added to give a final concentration of 1 mmol/l, followed by further culturing at 30°C for 4 hours. The resulting culture was centrifuged to obtain wet cells.

A reaction mixture (20 ml, pH 7.2) comprising 200
15 g/l wet cells, 50 g/l glucose, 5 g/l phytic acid (diluted to neutrality with 33% conc. sodium hydroxide solution), 15 g/l potassium dihydrogenphosphate, 5 g/l magnesium sulfate heptahydrate, 4 g/l Nymeen S-215, 10 ml/l xylene, 200 mmol/l L-Ala and 200 mmol/l L-Gln was put in a 50-ml
20 beaker, and reaction was carried out at 32°C at 900 rpm for 2 hours. During the reaction, the pH of the reaction mixture was maintained at 7.2 by using 2 mol/l potassium hydroxide.

The reaction product was analyzed by the same method
25 as in Experimental Example 3, whereby it was confirmed that 25 mg/l L-Ala-L-Gln was accumulated.

Experimental Example 6

Cloning of Genes Corresponding to the ywfe Gene from
30 Various Microorganisms of the Genus Bacillus and Analysis Thereof

On the basis of the nucleotide sequence shown in SEQ ID NO: 26, genes corresponding to the ywfe gene which exist in Bacillus subtilis ATCC 15245, ATCC 6633, IAM 1213,
35 IAM 1107, IAM 1214, ATCC 9466, IAM 1033 and ATCC 21555, Bacillus amyloliquefaciens IFO 3022 and Bacillus pumilus

NRRL B-12025 were obtained in the following manner.

That is, Bacillus subtilis ATCC 15245, ATCC 6633, IAM 1213, IAM 1107, IAM 1214, ATCC 9466, IAM 1033 and ATCC 21555, Bacillus amyloliquefaciens IFO 3022 and Bacillus pumilus NRRL B-12025 were respectively inoculated into LB medium and subjected to static culture overnight at 30°C. After the culturing, the chromosomal DNAs of the respective microorganisms were isolated and purified according to the method using saturated phenol described in Current Protocols in Molecular Biology.

By using a DNA synthesizer (Model 8905, PerSeptive Biosystems, Inc.), DNAs having the nucleotide sequences shown in SEQ ID NOS: 37 and 38 (hereinafter referred to as primer C and primer D, respectively) were synthesized. Primer C has a sequence containing a region upstream of the initiation codon of ywfE gene of the chromosomal DNA of Bacillus subtilis 168, and primer D has a sequence complementary to a sequence containing a region downstream of the termination codon of ywfE gene.

PCR was carried out using each of the chromosomal DNAs of Bacillus subtilis ATCC 15245, ATCC 6633, IAM 1213, IAM 1107, IAM 1214, ATCC 9466, IAM 1033 and ATCC 21555 and Bacillus amyloliquefaciens IFO 3022 as a template and the above primer C and primer D as a set of primers. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for one minute, reaction at 55°C for 2 minutes and reaction at 72°C for 3 minutes, using 40 µl of a reaction mixture comprising 0.1 µg of the chromosomal DNA, 0.5 µmol/l each of the primers, 2.5 units of Pfu DNA polymerase, 4 µl of buffer for Pfu DNA polymerase (10 x) and 200 µmol/l each of dNTPs.

One-tenth of each of the resulting reaction mixtures was subjected to agarose gel electrophoresis to confirm that a ca. 1.4 kb fragment corresponding to the ywfE gene fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of

phenol/chloroform saturated with TE. The resulting solution was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80°C for 30 minutes. The resulting solution
5 was centrifuged, and the obtained DNA precipitate was dissolved in 20 μ l of TE.

Each of the thus obtained 1.4 kb DNA fragments derived from the chromosomal DNAs of the respective strains and pCR-blunt (Invitrogen Corp.) were subjected to
10 ligation reaction using a ligation kit at 16°C for 16 hours.

Escherichia coli NM522 was transformed using each ligation reaction mixture according to the method using calcium ion, spread on LB agar medium containing 50 μ g/ml
15 ampicillin, and cultured overnight at 30°C.

A plasmid was extracted from a colony of each transformant that grew on the medium according to a known method and the structure of each plasmid was analyzed using restriction enzymes. As a result, it was confirmed
20 that the following plasmids containing a gene corresponding to the ywfE gene were obtained: pYWFE1 (derived from ATCC 15245, DNA having the nucleotide sequence shown in SEQ ID NO: 65), pYWFE2 (derived from ATCC 6633, DNA having the nucleotide sequence shown in SEQ
25 ID NO: 27), pYWFE3 (derived from IAM 1213, DNA having the nucleotide sequence shown in SEQ ID NO: 28), pYWFE4 (derived from IAM 1107, DNA having the nucleotide sequence shown in SEQ ID NO: 29), pYWFE5 (derived from IAM 1214, DNA having the nucleotide sequence shown in SEQ ID NO: 30),
30 pYWFE6 (derived from ATCC 9466, DNA having the nucleotide sequence shown in SEQ ID NO: 26), pYWFE7 (derived from IAM 1033, DNA having the nucleotide sequence shown in SEQ ID NO: 65), pYWFE8 (derived from ATCC 21555, DNA having the nucleotide sequence shown in SEQ ID NO: 31) and pYWFE9
35 (derived from IFO 3022, DNA having the nucleotide sequence shown in SEQ ID NO: 32).

On the other hand, a gene corresponding to ywfE gene derived from Bacillus pumilus NRRL B-12025 (DNA having the nucleotide sequence shown in SEQ ID NO: 64) was obtained in the following manner.

5 PCR was carried out using the chromosomal DNA of the NRRL B-12025 strain prepared above as a template and DNAs respectively consisting of the nucleotide sequences shown in SEQ ID NOS: 66 and 67 as a set of primers. That is, PCR was carried out by 30 cycles, one cycle consisting of
10 reaction at 98°C for 5 seconds, reaction at 55°C for 30 seconds and reaction at 72°C for one minute, using 50 μ l of a reaction mixture comprising 0.1 μ g of the chromosomal DNA, 0.5 μ mol/l each of the primers, 2.5 units of Z-taq polymerase (Takara Bio Inc.), 5 μ l of
15 buffer for Z-taq polymerase (10 x) (Takara Bio Inc.) and 200 μ mol/l each of dNTPs.

One-tenth of the resulting reaction mixture was subjected to agarose gel electrophoresis to confirm that a ca. 0.8 kb fragment was amplified. Then, the remaining
20 reaction mixture was mixed with an equal amount of phenol/chloroform saturated with TE. The resulting mixture was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80°C for 30 minutes. The resulting solution
25 was centrifuged, and the obtained DNA precipitate was dissolved in 20 μ l of TE.

The thus obtained 0.8 kb fragment derived from the chromosomal DNA and pGEM T-easy (Promega Corp.) were subjected to ligation reaction using a ligation kit at
30 16°C for 16 hours.

Escherichia coli DH5 α was transformed using the reaction mixture according to the method using calcium ion, spread on LB agar medium containing 50 μ g/ml ampicillin, and cultured overnight at 30°C.

35 A plasmid was extracted from the transformant obtained above and the nucleotide sequence of the ca. 0.8

kb DNA insert was determined, whereby a sequence from nucleotides 358 to 1160 in the nucleotide sequence shown in SEQ ID NO: 64 was confirmed.

The above plasmid was cleaved with EcoRI and then
5 subjected to agarose gel electrophoresis to separate a DNA fragment. The DNA fragment was purified using GENECLAN II Kit, and about 0.5 μ g of the purified DNA fragment was DIG-labeled using DIG-High Prime DNA Labeling & Detection Starter Kit I (Roche Diagnostics Corp.) according to the
10 instructions attached thereto.

Southern analysis of the chromosomal DNA of the NRRL B-12025 strain was carried out using the DIG-labeled DNA obtained above.

The chromosomal DNA of the NRRL B-12025 strain was
15 completely digested with BamHI, EcoRI, HindIII, KpnI, PstI, SacI, SalI and SphI, respectively, and subjected to agarose gel electrophoresis to separate DNA fragments, followed by transfer to nylon membrane plus charge (Roche Diagnostics Corp.) according to an ordinary method.

20 After the DNA fragments were fixed on the nylon membrane by UV irradiation, Southern hybridization was carried out using the above probe DNA and the nylon membrane.

The hybridization was carried out by contacting the
25 nylon membrane with the probe DNA at 65°C for 16 hours, washing the nylon membrane twice with a solution consisting of 0.1% SDS and 2 x SSC at room temperature for 5 minutes, and further washing the membrane twice with a solution consisting of 0.1% SDS and 0.5 x SSC at 65°C for
30 15 minutes. The other operations and conditions and detection of the hybridized DNA were carried out according to the instructions attached to the above-mentioned DIG-High Prime DNA Labeling & Detection Starter Kit I.

As a result, color development was observed at
35 around 3.5 kbp of the fragments completely digested with HindIII and PstI.

Subsequently, the chromosomal DNA of the NRRL B-12025 strain was completely digested with HindIII and PstI, respectively, and subjected to agarose gel electrophoresis to separate DNA fragments. From the respective
5 restriction enzyme-digested DNAs, 3-4 kbp fragments were purified using GENECLAN II Kit, followed by autocyclization using a ligation kit.

On the basis of the nucleotide sequence of the 0.8 kb DNA fragment determined above, the nucleotide sequences
10 shown in SEQ ID NOS: 71 and 72 were designed and synthesized, and they were used in PCR using the cyclized DNA obtained above as a template. PCR was carried out by 30 cycles, one cycle consisting of reaction at 98°C for 5 seconds, reaction at 55°C for 30 seconds and reaction at
15 72°C for 3 minutes and 30 seconds, using 50 µl of a reaction mixture comprising 10 ng of the cyclized DNA, 0.5 µmol/l each of the primers, 2.5 units of pyrobest polymerase (Takara Bio Inc.), 5 µl of buffer for pyrobest polymerase (10 x) (Takara Bio Inc.) and 200 µmol/l each
20 of dNTPs.

One-tenth of the resulting reaction mixture was subjected to agarose gel electrophoresis to confirm that a ca. 3.0 kb fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of
25 phenol/chloroform saturated with TE. The resulting mixture was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80°C for 30 minutes. The resulting solution was centrifuged, and the obtained DNA precipitate was
30 dissolved in 20 µl of TE.

The thus obtained DNA fragment and Zero Blunt PCR Cloning Kit (Invitrogen Corp.) were subjected to ligation reaction using a ligation kit.

Escherichia coli NM522 was transformed using the
35 reaction mixture according to the method using calcium ion, spread on LB agar medium containing 50 µg/ml ampicillin,

and cultured overnight at 30°C.

A plasmid was extracted from a colony of the transformant that grew on the medium according to a known method and the structure of the plasmid was analyzed using
5 restriction enzymes. As a result, it was confirmed that plasmid pYWFE10 (derived from NRRL B-12025, DNA having the nucleotide sequence shown in SEQ ID NO: 64) containing a gene corresponding to the ywfE gene was obtained.

The nucleotide sequences of the genes corresponding
10 to the ywfE gene which are respectively contained in the plasmids pYWFE1 to pYWFE10 obtained above were determined using 373A DNA Sequencer.

The amino acid sequences of the proteins encoded by the genes respectively contained in pYWFE1, pYWFE6 and
15 pYWFE7 were identical with the amino acid sequence of the protein encoded by the ywfE gene, whereas those of the proteins encoded by the genes respectively contained in pYWFE2, pYWFE3, pYWFE4, pYWFE5, pYWFE8, pYWFE9 and pYWFE10 were different from the amino acid sequence of the protein
20 encoded by the ywfE gene.

The amino acid sequences of the proteins encoded by the genes corresponding to the ywfE gene which are contained in pYWFE2, pYWFE3, pYWFE4, pYWFE5, pYWFE8, pYWFE9 and pYWFE10, and pYWFE1 and pYWFE7 are shown in SEQ
25 ID NOS: 20 to 25, 68 and 19, respectively, and the nucleotide sequences of these genes are shown in SEQ ID NOS: 27 to 32, 65 and 26, respectively.

Experimental Example 7

30 Purification of C-Terminal His-Tagged Recombinant Dipeptide Synthetase

PCR was carried out using each of the chromosomal DNAs of Bacillus subtilis ATCC 15245, ATCC 6633, IAM 1213, IAM 1107, IAM 1214, ATCC 9466, IAM 1033 and ATCC 21555 and
35 Bacillus amyloliquefaciens IFO 3022 as a template and primer A and primer B described in Experimental Example 1

as a set of primers. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for one minute, reaction at 55°C for 2 minutes and reaction at 72°C for 3 minutes, using 40 μ l of a reaction mixture
5 comprising 0.1 μ g of the chromosomal DNA, 0.5 μ mol/l each of the primers, 2.5 units of Pfu DNA polymerase, 4 μ l of buffer for Pfu DNA polymerase (10 x) and 200 μ mol/l each of dNTPs.

When the chromosomal DNA of Bacillus pumilus NRRL B-
10 12025 was used as a template, PCR was carried out using DNAs respectively having the nucleotide sequences shown in SEQ ID NOS: 69 and 70 as a set of primers under the same conditions as above.

One-tenth of each of the resulting reaction mixtures
15 was subjected to agarose gel electrophoresis to confirm that a ca. 1.4 kb DNA fragment corresponding to the ywfE fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of phenol/chloroform saturated with TE. The resulting
20 mixture was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80°C for 30 minutes. The resulting solution was centrifuged, and the obtained DNA precipitate was dissolved in 20 μ l of TE.

Each of the thus obtained solutions (5 μ l) was
25 subjected to reaction to cleave the amplified DNA with restriction enzymes NcoI and BamHI. DNA fragments were separated by agarose gel electrophoresis, and a 1.4 kb DNA fragment containing a gene corresponding to the ywfE gene
30 was recovered using GENECLAN II Kit.

Subsequently, 0.2 μ g of the C-terminal His-tagged recombinant expression vector pQE60 was cleaved with
restriction enzymes NcoI and BamHI. DNA fragments were separated by agarose gel electrophoresis, and a 3.4 kb DNA
35 fragment was recovered in the same manner as above.

Each of the 1.4 kb DNA fragments containing a gene

corresponding to the ywfE gene of Bacillus subtilis 168 and the 3.4 kb DNA fragment obtained above were subjected to ligation reaction using a ligation kit at 16°C for 16 hours. Escherichia coli NM522 was transformed using each
5 ligation reaction mixture according to the method using calcium ion, spread on LB agar medium containing 50 µg/ml ampicillin, and cultured overnight at 30°C.

A plasmid was extracted from a colony of each transformant that grew on the medium according to a known
10 method and the structure of each plasmid was analyzed using restriction enzymes. As a result, it was confirmed that the following C-terminal His-tagged gene expression vectors were obtained: pQE60ywfE1 (a vector containing the gene derived from ATCC 15245), pQE60ywfE2 (a vector
15 containing the gene derived from ATCC 6633), pQE60ywfE3 (a vector containing the gene derived from IAM 1213), pQE60ywfE4 (a vector containing the gene derived from IAM 1107), pQE60ywfE5 (a vector containing the gene derived from IAM 1214), pQE60ywfE6 (a vector containing the gene
20 derived from ATCC 9466), pQE60ywfE7 (a vector containing the gene derived from IAM 1033), pQE60ywfE8 (a vector containing the gene derived from ATCC 21555), pQE60ywfE9 (a vector containing the gene derived from IFO 3022) and pQE60ywfE10 (a vector containing the gene derived from
25 NRRL B-12025).

Escherichia coli NM522/pQE60ywfE1 to NM522/pQE60ywfE10 strains obtained above were respectively inoculated into 8 ml of LB medium containing 50 µg/ml ampicillin in a test tube, and cultured at 28°C for 17
30 hours. Each of the resulting cultures was inoculated into 50 ml of LB medium containing 50 µg/ml ampicillin in a 250-ml Erlenmeyer flask, and cultured at 30°C for 3 hours. Then, IPTG was added to give a final concentration of 1 mmol/l, followed by further culturing at 30°C for 4 hours.
35 The resulting culture was centrifuged to obtain wet cells, and His-tagged recombinant enzymes were purified from the

respective wet cells using HisTrap according to the instructions attached thereto.

Experimental Example 8

5 Production of Dipeptides Using Purified Enzymes

Reaction mixtures (0.1 ml each) comprising 0.04 mg of the respective recombinant enzymes obtained in Experimental Example 7, 100 mmol/l Tris-HCl (pH 8.0), 60 mmol/l magnesium chloride, 60 mmol/l ATP, 30 mmol/l L-Ala
10 and 30 mmol/l L-Gln were prepared, and reactions were carried out at 37°C for 16 hours.

After the completion of reactions, the reaction mixtures were analyzed by the method described in Experimental Example 3, whereby it was confirmed that 3.0
15 to 3.5 g/l L-Ala-L-Gln and 0.25 to 0.3 g/l L-Ala-L-Ala were formed and accumulated.

When ATP was excluded from the compositions of the above reaction mixtures, L-Ala-L-Gln or L-Ala-L-Ala was not formed at all.

20 The above results revealed that all of the products of the genes obtained in Experimental Example 7 have the activity to produce L-Ala-L-Gln and L-Ala-L-Ala from L-Ala and L-Gln in the presence of ATP.

25 Experimental Example 9

Construction of Escherichia coli for Enhanced Expression of the ywfE Gene

By using a DNA synthesizer (Model 8905, PerSeptive Biosystems, Inc.), DNAs having the sequences shown in SEQ
30 ID NOS: 60 to 63 (hereinafter referred to as primer E, primer F, primer G and primer H, respectively) were synthesized. The sequence of SEQ ID NO: 60 is a sequence wherein a sequence containing the XhoI recognition sequence is added to the 5' end of a region containing the
35 Shine-Dalgarno sequence (ribosome binding sequence) of ywfE gene on the plasmid pQE60ywfE. The sequence of SEQ

ID NO: 61 is a sequence wherein a sequence containing the BamHI recognition sequence is added to the 5' end of a sequence complementary to a sequence containing the termination codon of ywfE gene. The sequence of SEQ ID
5 NO: 62 is a sequence wherein a sequence containing the EcoRI recognition sequence is added to the 5' end of the sequence of trp promoter region of expression vector pTrS30 containing trp promoter. The sequence of SEQ ID
10 NO: 63 is a sequence wherein a sequence containing the XhoI recognition sequence is added to the 5' end of a sequence complementary to the sequence of trp promoter region of expression vector pTrS30 containing trp promoter.

A ywfE gene fragment and a trp promoter region fragment were amplified by PCR using the above primers E
15 and F and primers G and H as a set of primers, respectively, and the plasmid pQE60ywfE as a template. PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for one minute, reaction at 55°C for 2 minutes and reaction at 72°C for 3 minutes, using 40 μ l
20 of a reaction mixture comprising 10 ng of pQE60ywfE, 0.5 μ mol/l each of the primers, 2.5 units of Pfu DNA polymerase, 4 μ l of buffer for Pfu DNA polymerase (10 x) and 200 μ mol/l each of dNTPs.

One-tenth of each of the resulting reaction mixtures
25 was subjected to agarose gel electrophoresis to confirm that a ca. 1.4 kb fragment corresponding to the ywfE gene fragment and a ca. 0.3 kb fragment corresponding to the trp promoter region fragment were respectively amplified in the PCR using primer E and primer F and the PCR using
30 primer G and primer H. Then, the remaining reaction mixture was mixed with an equal amount of phenol/chloroform saturated with TE. The resulting solution was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed
35 to stand at -80°C for 30 minutes. The resulting solution was centrifuged, and the obtained DNA was dissolved in 20

μl of TE.

The thus obtained DNA solutions (5 μl each) were respectively subjected to reaction to cleave the DNA amplified using primer E and primer F with restriction enzymes XhoI and BamHI and to reaction to cleave the DNA amplified using primer G and primer H with restriction enzymes EcoRI and XhoI. DNA fragments were separated by agarose gel electrophoresis, and a 1.4 kb fragment containing ywfE gene and a 0.3 kb fragment containing trp promoter region were respectively recovered using GENECLAN II Kit.

pTrs30 (a trp promoter-containing expression vector, 0.2 μg) was cleaved with restriction enzymes EcoRI and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 4.5 kb DNA fragment was recovered in the same manner as above.

The 1.4 kb fragment containing the ywfE gene, the 0.3 kb fragment containing trp promoter region and the 4.5 kb DNA fragment obtained above were subjected to ligation reaction using a ligation kit at 16°C for 16 hours.

Escherichia coli NM522 was transformed using the reaction mixture according to the method using calcium ion, spread on LB agar medium containing 50 μg/ml ampicillin, and cultured overnight at 30°C.

A plasmid was extracted from a colony of the transformant that grew on the medium according to a known method, whereby expression vector pPE56 containing the ywfE gene in a downstream position of the trp promoter was obtained. The structure of the vector was confirmed by digestion with restriction enzymes (Fig. 2).

Certain embodiments of the present invention are illustrated in the following examples. These examples are not to be construed as limiting the scope of the invention.

Example 1

Preparation of Strains Having pepD gene, pepN gene, pepB gene, pepA gene and dpp Operon Deletions

5 Strains in which specific genes on Escherichia coli chromosomal DNA are deleted were prepared according to the method utilizing the homologous recombination system of lambda phage [Proc. Natl. Acad. Sci. USA, 97, 6641-6645 (2000)].

10 Plasmids pKD46, pKD3 and pCP20 used below were prepared by extraction from Escherichia coli strains carrying them which were obtained from Escherichia coli Genetic Stock Center, Yale University, U.S.A.

(1) Cloning of DNA Fragments for Gene Deletion

15 For the purpose of deleting the following genes existing on the chromosomal DNA of Escherichia coli K12, DNAs having nucleotide sequences homologous to 36-bp nucleotide sequences that lie upstream and downstream of the respective genes to be deleted on the chromosomal DNA
20 of Escherichia coli K12 and the nucleotide sequence shown in SEQ ID NO: 39 which is recognized by yeast-derived Flp recombinase were synthesized using a DNA synthesizer (Model 8905, PerSeptive Biosystems, Inc.). The genes to be deleted are pepD gene having the nucleotide sequence
25 shown in SEQ ID NO: 10, pepN gene having the nucleotide sequence shown in SEQ ID NO: 11, pepB gene having the nucleotide sequence shown in SEQ ID NO: 12, pepA gene having the nucleotide sequence shown in SEQ ID NO: 13, dppA gene having the nucleotide sequence shown in SEQ ID
30 NO: 14, dppB gene having the nucleotide sequence shown in SEQ ID NO: 15, dppC gene having the nucleotide sequence shown in SEQ ID NO: 16, dppD gene having the nucleotide sequence shown in SEQ ID NO: 17 and dppF gene having the nucleotide sequence shown in SEQ ID NO: 18. In the case
35 of dppA gene, dppB gene, dppC gene, dppD gene and dppF gene, which form an operon, DNAs having nucleotide

sequences homologous to the nucleotide sequences that lie upstream and downstream of the operon were synthesized.

That is, DNAs consisting of the following nucleotide sequences were synthesized as respective sets of primers for amplification of DNA fragments for gene deletion: SEQ ID NOS: 40 and 41 for pepD gene deletion; SEQ ID NOS: 42 and 43 for pepN gene deletion; SEQ ID NOS: 44 and 45 for pepA gene deletion; SEQ ID NOS: 46 and 47 for pepB gene deletion; and SEQ ID NOS: 48 and 49 for dpp operon deletion.

Subsequently, PCR was carried out using each set of the above synthetic DNAs as a set of primers and pKD3 DNA as a template. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for one minute, reaction at 55°C for 2 minutes and reaction at 72°C for 3 minutes, using 40 μ l of a reaction mixture comprising 10 ng of the plasmid DNA, 0.5 μ mol/l each of the primers, 2.5 units of Pfu DNA polymerase (Stratagene), 4 μ l of buffer for Pfu DNA polymerase (10 x) (Stratagene) and 200 μ mol/l each of deoxyNTPs (dATP, dGTP, dCTP and dTTP).

One-tenth of each of the resulting reaction mixtures was subjected to agarose gel electrophoresis to confirm that the desired fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of phenol/chloroform (1 vol/1 vol) saturated with TE [10 mmol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA].

The resulting mixture was centrifuged, and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80°C for 30 minutes, followed by centrifugation. By this procedure, chloramphenicol resistance gene-containing DNA fragments for deletion of pepD gene, pepN gene, pepB gene, pepA gene and dpp operon were obtained.

(2) Preparation of Escherichia coli JM101 Having pepD Gene Deletion

Escherichia coli JM101 was transformed with pKD46, spread on LB agar medium containing 100 mg/l ampicillin, and cultured at 30°C to select a transformant.

The plasmid pKD46 carries inserted λ Red recombinase gene and is designed so that the expression of the gene is induced by L-arabinose. Accordingly, when the Escherichia coli grown in the presence of L-arabinose is transformed using a linear DNA, homologous recombination occurs with high frequency. Further, as pKD46 has a thermosensitive replication origin, curing of the plasmid can be readily caused by culturing the strain at 42°C.

The chloramphenicol resistance gene-containing DNA fragment for pepD gene deletion obtained above was introduced into Escherichia coli JM101/pKD46 obtained by culturing in the presence of 10 mmol/l L-arabinose and 50 μ g/ml ampicillin by electroporation. The resulting cells were spread on LB agar medium (10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 5 g/l sodium chloride and 15 g/l agar) containing 25 mg/l chloramphenicol and cultured at 30°C to select a transformant in which the chloramphenicol resistance gene-containing DNA fragment for pepD gene deletion was integrated into the chromosomal DNA of Escherichia coli JM101 by homologous recombination.

The selected chloramphenicol-resistant strain was inoculated onto LB agar medium containing 25 mg/l chloramphenicol and cultured at 42°C for 14 hours, followed by single colony isolation. Replicas of the obtained colonies were made on LB agar medium containing 25 mg/l chloramphenicol and LB agar medium containing 100 mg/l ampicillin, followed by culturing at 37°C. By selecting a colony showing chloramphenicol resistance and ampicillin sensitivity, a pKD46-cured strain was obtained.

The pKD46-cured strain thus obtained was transformed using pCP20, followed by selection on LB agar medium containing 100 mg/l ampicillin to obtain a pKD46-cured strain carrying pCP20.

The plasmid pCP20 carries inserted yeast-derived Flp recombinase gene and is designed so that the expression of the gene is induced at a temperature of 42°C.

5 The chloramphenicol resistance gene-containing DNA fragments for deletion of pepD gene, pepN gene, pepB gene, pepA gene and dpp operon prepared above contain nucleotide sequences recognized by Flp recombinase at both termini of the chloramphenicol resistance gene. Therefore, the resistance gene can be readily deleted by homologous
10 recombination catalyzed by Flp recombinase.

Further, as pCP20 has a thermosensitive replication origin, expression of Flp recombinase and curing of pCP20 can be simultaneously induced by culturing the pCP20-carrying strain at 42°C.

15 The pCP20-carrying pKD46-cured strain obtained above was inoculated onto drug-free LB agar medium and cultured at 42°C for 14 hours, followed by single colony isolation. Replicas of the obtained colonies were made on drug-free LB agar medium, LB agar medium containing 25 mg/l
20 chloramphenicol and LB agar medium containing 100 mg/l ampicillin, followed by culturing at 30°C. Then, colonies showing chloramphenicol sensitivity and ampicillin sensitivity were selected.

Chromosomal DNAs were prepared from the respective
25 strains selected above according to an ordinary method [Seibutsukogaku Jikkensho (Experiments in Biotechnology), edited by The Society for Biotechnology, Japan, p. 97-98, Baifukan (1992)]. PCR was carried out using, as a set of primers, DNAs having the nucleotide sequences shown in SEQ
30 ID NOS: 50 and 51 which were designed based on an inner nucleotide sequence of the pepD gene to be deleted, and using each of the chromosomal DNAs as a template. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for one minute, reaction at 55°C for 2
35 minutes and reaction at 72°C for 3 minutes, using 40 µl of a reaction mixture comprising 0.1 µg of the

chromosomal DNA, 0.5 μ mol/l each of the primers, 2.5 units of Pfu DNA polymerase, 4 μ l of buffer for Pfu DNA polymerase (10 x) and 200 μ mol/l each of deoxyNTPs.

5 A strain with which no amplified DNA fragment was detected in the above PCR was identified as a strain having pepD gene deletion and was designated as Escherichia coli JPD1.

(3) Preparation of a Strain in Which the pepD Gene and pepN Gene on the Chromosomal DNA of Escherichia coli JM101 are Deleted

Escherichia coli JPD1 obtained in the above (2) was transformed with pKD46, spread on LB agar medium containing 100 mg/l ampicillin, and cultured at 30°C to
15 select a transformant. The chloramphenicol resistance gene-containing DNA fragment for pepN gene deletion was introduced into the obtained transformant (Escherichia coli JPD1/pKD46) by electroporation to obtain a transformant in which the chloramphenicol resistance gene-
20 containing DNA fragment for pepN gene deletion was integrated into the chromosomal DNA of Escherichia coli JPD1/pKD46 by homologous recombination.

Subsequently, the same procedure as in the above (2) was carried out to obtain a strain in which the
25 chloramphenicol resistance gene was deleted from the chromosomal DNA, which was designated as Escherichia coli JPDN2.

(4) Preparation of Strains in Which pepN Gene, pepA Gene, pepB Gene or dpp Operon on the Chromosomal DNA of
30 Escherichia coli JM101 is Deleted and Strains Having Multiple Gene Deletion

The strains having pepN gene, pepA gene, pepB gene or dpp operon deletion were prepared according to the same
35 procedure as in the above (2) using the respective chloramphenicol resistance gene-containing DNA fragments

for gene or operon deletion prepared in the above (1).

Acquisition of the strains having gene deletions by the above method was confirmed by carrying out PCR in the same manner as in the above (2) using, as sets of primers, DNAs having the nucleotide sequences shown in SEQ ID NOS: 52 to 59 which were designed and synthesized based on inner nucleotide sequences of the respective genes to be deleted. That is, DNAs having the following nucleotide sequences were used as respective sets of primers for the confirmation of gene deletion: SEQ ID NOS: 52 and 53 for pepN gene deletion; SEQ ID NOS: 54 and 55 for pepA gene deletion; SEQ ID NOS: 56 and 57 for pepB gene deletion; and SEQ ID NOS: 58 and 59 for dpp operon deletion.

The thus obtained dpp operon-deleted strain, pepN gene-deleted strain, pepA gene-deleted strain and pepB gene-deleted strain were designated as Escherichia coli JDPP1, Escherichia coli JPN1, Escherichia coli JPA1 and Escherichia coli JPB7, respectively.

Further, strains having multiple gene deletions, i.e., deletions of two or more genes or operon selected from the group consisting of pepD gene, pepN gene, pepA gene, pepB gene and dpp operon were prepared according to the method of the above (3). Acquisition of the strains having multiple gene deletions was confirmed by PCR similar to that in the above (2). The thus obtained double gene-deleted strain having pepD gene and dpp operon deletions was designated as Escherichia coli JPDP49, triple gene-deleted strain having pepB gene, pepD gene and pepN gene deletions as Escherichia coli JPDNB43, triple gene-deleted strain having pepD gene, pepN gene and dpp operon deletions as Escherichia coli JPNDDP36, quadruple gene-deleted strain having pepA gene, pepD gene, pepN gene and dpp operon deletions as Escherichia coli JPNDA5, and quadruple gene-deleted strain having pepB gene, pepD gene, pepN gene and dpp operon deletions as Escherichia coli JPNDBP7. The genes deleted in the gene-deleted strains

are shown in Table 2.

Table 2

Strain	Deleted gene
JM101	none
JDPP1	dpp operon
JPN1	pepN
JPA1	pepA
JPB7	pepB
JPD1	pepD
JPDN2	pepD, pepN
JPNDB43	pepB, pepD, pepN
JPDP49	pepD, dpp operon
JPNDDP36	pepD, pepN, dpp operon
JPNDAP5	pepA, pepD, pepN, dpp operon
JPNDBP7	pepB, pepD, pepN, dpp operon

5 Example 2

Evaluation of Productivity of L-Alanyl-L-glutamine (hereinafter referred to as AlaGln) and L-Alanyl-L-alanine (hereinafter referred to as AlaAla) by Escherichia coli Strains in Which Peptidase and Peptide-transporting

10 Activities are Lost

The strains having deletions of genes encoding various peptidase genes and an operon encoding peptide-transporting protein which were obtained in Example 1 were transformed using the plasmid pPE56 constructed in
15 Experimental Example 8 to obtain ampicillin-resistant transformants.

Each of the obtained transformants was inoculated into 8 ml of LB medium containing 50 μ g/ml ampicillin in a test tube and cultured at 28°C for 17 hours. The
20 resulting culture was inoculated into 8 ml of a production medium [16 g/l dipotassium hydrogenphosphate, 14 g/l potassium dihydrogenphosphate, 5 g/l ammonium sulfate, 1 g/l citric acid (anhydrous), 0.5 g/l Casamino acid (Difco),

1 g/l L-Pro, 2.5 g/l L-Ala, 2.5 g/l L-Gln, 10 g/l glucose,
10 mg/l vitamin B₁, 25 mg/l magnesium sulfate heptahydrate
and 50 mg/l ferrous sulfate heptahydrate; pH adjusted to
7.2 with 10 mol/l sodium hydroxide solution; L-Gln was
5 added after sterilization by filtration of a 10-fold conc.
solution; glucose, vitamin B₁, magnesium sulfate
heptahydrate and ferrous sulfate heptahydrate were added
after separate autoclaving] containing 100 µg/ml
ampicillin in a test tube in an amount of 1% and cultured
10 at 30°C for 24 hours. The resulting culture was
centrifuged to obtain a culture supernatant.

The product in the culture supernatant was
derivatized by the F-moc method and then analyzed by HPLC.
The HPLC analysis was carried out using ODS-HG5 (Nomura
15 Kagaku Co., Ltd.) as a separation column and solution A (6
ml/l acetic acid and 20% (v/v) acetonitrile, pH adjusted
to 4.8 with triethylamine) and solution B (6 ml/l acetic
acid and 70% (v/v) acetonitrile, pH adjusted to 4.8 with
triethylamine) as eluting solutions. The ratio of
20 solution A to solution B was 8:2 during the first 5
minutes of elution and thereafter changed with a linear
gradient so that the ratio became 1:1 at 20 minutes after
the start of elution. The results of analysis are shown
in Table 3.

Table 3

Strain	Deleted gene	AlaGln (g/l)	AlaAla (g/l)
JM101	none	0	0
JDPP1	dpp operon	0.02	0.01
JPN1	pepN	0.01	0.01
JPA1	pepA	0.01	0.01
JPB7	pepB	0.01	0.01
JPD1	pepD	0.01	0.01
JPDN2	pepD, pepN	0.02	0.03
JPNDB43	pepB, pepD, pepN	0.05	0.12
JPDP49	pepD, dpp operon	0.11	0.08
JPNDDP36	pepD, pepN, dpp operon	0.16	0.21
JPNDAP5	pepA, pepD, pepN, dpp operon	0.28	0.26
JPNDBP7	pepB, pepD, pepN, dpp operon	0.43	0.22

As can be seen from Table 3, small amounts of dipeptides were formed and accumulated by use of the microorganisms having deletions of two or less kinds of peptidase genes or one kind of peptide-transporting protein gene, whereas the amounts of dipeptides formed and accumulated were greatly increased by use of the microorganisms having deletions of one or more kinds of peptidase genes and one kind of peptide-transporting protein gene or microorganisms having deletions of three or more kinds of peptidase genes.

Example 3

Evaluation of Productivity of L-Alanyl-L-valine (hereinafter referred to as AlaVal) by Escherichia coli Strains in Which Peptidase and Peptide-transporting Protein Activities are Lost

Similarly to Example 2, the Escherichia coli strains having deletions of various peptidase genes and an operon encoding a peptide-transporting protein were transformed using pPE56. Each of the obtained transformants was

inoculated into 8 ml of LB medium containing 50 μ g/ml
 ampicillin in a test tube and cultured at 28°C for 17
 hours. The resulting culture was inoculated into 8 ml of
 a production medium [16 g/l dipotassium hydrogenphosphate,
 5 14 g/l potassium dihydrogenphosphate, 5 g/l ammonium
 sulfate, 1 g/l citric acid (anhydrous), 0.5 g/l Casamino
 acid (Difco), 1 g/l L-Pro, 2.5 g/l L-Ala, 2.5 g/l L-Val,
 10 g/l glucose, 10 mg/l vitamin B₁, 25 mg/l magnesium
 sulfate heptahydrate and 50 mg/l ferrous sulfate
 10 heptahydrate; pH adjusted to 7.2 with 10 mol/l sodium
 hydroxide solution; glucose, vitamin B₁, magnesium sulfate
 heptahydrate and ferrous sulfate heptahydrate were added
 after separate autoclaving] containing 100 μ g/ml
 ampicillin in a test tube in an amount of 1% and cultured
 15 at 30°C for 24 hours. The resulting culture was
 centrifuged to obtain a culture supernatant.

The product in the culture supernatant was analyzed
 by the method described in Example 2. The results are
 shown in Table 4.

20

Table 4

Strain	Deleted gene	AlaVal (g/l)
JM101	none	0
JDPP1	dpp operon	0
JPN1	pepN	0
JPA1	pepA	0
JPB7	pepB	0
JPD1	pepD	0
JPDN2	pepD, pepN	0
JPNDB43	pepB, pepD, pepN	0.04
JPDP49	pepD, dpp operon	0.11
JPNDDP36	pepD, pepN, dpp operon	0.22
JPNDBP7	pepB, pepD, pepN, dpp operon	0.20

As can be seen from Table 4, the dipeptide was not

produced by use of the microorganisms having deletions of two or less kinds of peptidase genes or one kind of peptide-transporting protein gene, whereas the dipeptide was produced by use of the microorganisms having deletions of three or more kinds of peptidase genes or microorganisms having deletions of one or more kinds of peptidase genes and one kind of peptide-transporting protein gene.

10 Example 4

Evaluation of Productivity of Glycyl-L-glutamine (hereinafter referred to as GlyGln) by Escherichia coli Strains in Which Peptidase and Peptide-transporting Protein Activities are Lost

15 Similarly to Example 2, the strains having deletions of various peptidase genes and an operon encoding a peptide-transporting protein were transformed using pPE56. Each of the obtained transformants was inoculated into 8 ml of LB medium containing 50 μ g/ml ampicillin in a test tube and cultured at 28°C for 17 hours.

20 The resulting culture was inoculated into 8 ml of a production medium [16 g/l dipotassium hydrogenphosphate, 14 g/l potassium dihydrogenphosphate, 5 g/l ammonium sulfate, 1 g/l citric acid (anhydrous), 0.5 g/l Casamino acid (Difco), 1 g/l L-Pro, 2.5 g/l Gly, 2.5 g/l L-Gln, 10 g/l glucose, 10 mg/l vitamin B₁, 25 mg/l magnesium sulfate heptahydrate and 50 mg/l ferrous sulfate heptahydrate; pH adjusted to 7.2 with 10 mol/l sodium hydroxide solution; L-Gln was added after sterilization by filtration of a 10-fold conc. solution; glucose, vitamin B₁, magnesium sulfate heptahydrate and ferrous sulfate heptahydrate were added after separate autoclaving] containing 100 μ g/ml ampicillin in a test tube in an amount of 1% and cultured at 30°C for 24 hours. The resulting culture was centrifuged to obtain a culture supernatant.

The product in the culture supernatant was analyzed

by the method described in Example 2. The results are shown in Table 5.

Table 5

Strain	Deleted gene	GlyGln (g/l)
JM101	none	0
JDPP1	dpp operon	0
JPDN2	pepD, pepN	0
JPNDB43	pepB, pepD, pepN	0.01
JPNDDP36	pepD, pepN, dpp operon	0.02
JPNDBP7	pepB, pepD, pepN, dpp operon	0.03

5

As can be seen from Table 5, the dipeptide was not produced by use of the microorganisms having deletions of two or less kinds of peptidase genes or one kind of peptide-transporting protein gene, whereas the dipeptide was produced by use of the microorganisms having deletions of three or more kinds of peptidase genes or microorganisms having deletions of two or more kinds of peptidase genes and one kind of peptide-transporting protein gene.

15

SEQUENCE LISTING FREE TEXT

SEQ ID NO: 35 - Description of Artificial Sequence:
Synthetic DNA

20 SEQ ID NO: 36 - Description of Artificial Sequence:
Synthetic DNA

SEQ ID NO: 37 - Description of Artificial Sequence:
Synthetic DNA

25 SEQ ID NO: 38 - Description of Artificial Sequence:
Synthetic DNA

SEQ ID NO: 39 - Description of Artificial Sequence:
Synthetic DNA

SEQ ID NO: 40 - Description of Artificial Sequence:

Synthetic DNA
SEQ ID NO: 41 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 42 - Description of Artificial Sequence:
5 Synthetic DNA
SEQ ID NO: 43 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 44 - Description of Artificial Sequence:
Synthetic DNA
10 SEQ ID NO: 45 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 46 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 47 - Description of Artificial Sequence:
15 Synthetic DNA
SEQ ID NO: 48 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 49 - Description of Artificial Sequence:
Synthetic DNA
20 SEQ ID NO: 50 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 51 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 52 - Description of Artificial Sequence:
25 Synthetic DNA
SEQ ID NO: 53 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 54 - Description of Artificial Sequence:
Synthetic DNA
30 SEQ ID NO: 55 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 56 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 57 - Description of Artificial Sequence:
35 Synthetic DNA
SEQ ID NO: 58 - Description of Artificial Sequence:

Synthetic DNA
SEQ ID NO: 59 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 60 - Description of Artificial Sequence:
5 Synthetic DNA
SEQ ID NO: 61 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 62 - Description of Artificial Sequence:
Synthetic DNA
10 SEQ ID NO: 63 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 66 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 67 - Description of Artificial Sequence:
15 Synthetic DNA
SEQ ID NO: 69 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 70 - Description of Artificial Sequence:
Synthetic DNA
20 SEQ ID NO: 71 - Description of Artificial Sequence:
Synthetic DNA
SEQ ID NO: 72 - Description of Artificial Sequence:
Synthetic DNA

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

What is claimed is:

1. A process for producing a dipeptide, which comprises:

- allowing an enzyme source and one or more kinds of amino acids to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, or Ykfd;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide by condensation and ligation of two amino acids which are identical or different by producing a protein according to any one of [1] to [4] below:

[1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino

acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

[4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

wherein said microorganism comprises a recombinant DNA according to any one of [i] to [iv] below, ligated to a vector DNA:

[i] DNA encoding the protein defined in any one of [1] to [4] above;

[ii] DNA comprising the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65;

[iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65 under stringent conditions comprising hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1 to 2-fold SSC solution, and which encodes a protein having the activity to synthesize a dipeptide; and

[iv] DNA comprising a nucleotide sequence which has 80% or more identity to the full length nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

- recovering the dipeptide from the medium.

2. A process for producing a dipeptide, which comprises:

- allowing an enzyme source and one or more kinds of amino acids to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition, wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide by condensation and ligation of two amino acids which are identical or different by producing a protein according to any one of [1] to [4] below:

[1] a protein comprising the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68;

[2] a variant of the protein of [1] in which one to 20 amino acid residues are deleted, substituted or added in the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68, and which has the activity to synthesize a dipeptide;

[3] a protein comprising an amino acid sequence which has 65% or more identity to the full length of the amino acid sequence shown in any one of SEQ ID NOs: 19 to 25 and 68 and having the activity to synthesize a dipeptide; and

[4] a protein comprising an amino acid sequence which has 80% or more identity to the full length of the amino acid sequence shown in SEQ ID NO: 33 and having the activity to synthesize a dipeptide;

wherein said microorganism comprises a recombinant DNA according to any one of [i] to [iv] below, ligated to a vector DNA:

[i] DNA encoding the protein defined in any one of [1]

to [4] above;

[ii] DNA comprising the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65;

[iii] DNA which hybridizes with the complement of a DNA having the nucleotide sequence shown in any one of SEQ ID NOs: 26 to 32, 64 and 65 under stringent conditions comprising hybridization at 65°C in the presence of 0.7 to 1.0 mol/l sodium chloride and washing at 65°C with a 0.1 to 2-fold SSC solution, and which encodes a protein having the activity to synthesize a dipeptide; and

[iv] DNA comprising a nucleotide sequence which has 80% or more identity to the full length nucleotide sequence shown in SEQ ID NO: 34 and encoding a protein having the activity to synthesize a dipeptide;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

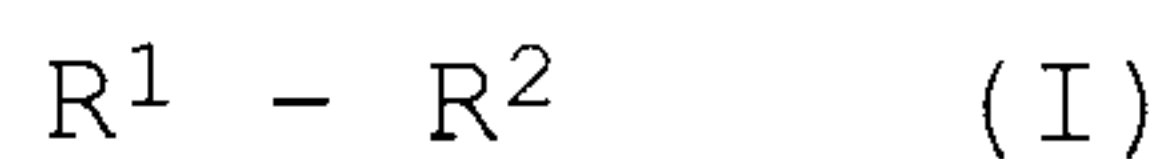
- recovering the dipeptide from the medium.

3. The process according to Claim 1, wherein the dipeptide is a dipeptide represented by the following formula (I):



wherein R^1 and R^2 , which may be the same or different, each represent an amino acid.

4. The process according to Claim 2, wherein the dipeptide is a dipeptide represented by the following formula (I):



wherein R^1 and R^2 , which may be the same or different, each represent an amino acid.

5. A process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, or Ykfd;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA; or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying,

or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and
- recovering the dipeptide from the medium.

6. A process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid ester and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid ester and an L-amino acid by producing proline iminopeptidase, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having proline iminopeptidase activity is ligated to a vector DNA; or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and
- recovering the dipeptide from the medium.

7. A process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid amide and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of one or more peptidases and one or more peptide-transporting proteins are reduced or lost by introduction of nucleotide deletion, substitution or addition into the genes encoding said one or more peptidases and peptide-transporting proteins in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidase is PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said peptide-transporting protein is DppA, DppB, DppC, DppD, DppF, OppA, OppB, OppC, OppD, OppF, YddO, YddP, YddQ, YddR, YddS, YbiK, MppA, SapA, SapB, SapC, SapD, SapF, DppA, DppB, DppC, DppD, DppE, OppA, OppB, OppC, OppD, OppF, AppA, AppB, AppC, AppD, AppF, YclF, or Ykfd;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

- recovering the dipeptide from the medium.

8. A process for producing a dipeptide, which comprises:

- allowing an enzyme source, an L-amino acid amide and an L-amino acid to be present in an aqueous medium, said enzyme source being:

(A) a culture of a microorganism in which the activities of three or more peptidases are reduced or lost by introduction of a nucleotide deletion, substitution or addition into the genes encoding said peptidases in the chromosomal DNA of the microorganism, relative to a corresponding microorganism lacking said nucleotide deletion, substitution or addition,

wherein said peptidases are PepA, PepB, PepD, PepN, PepP, PepQ, PepE, PepT, Dcp, IadA, AmpS, YbaC, YcdD, YjbG, YkvY, YqjE, or YwaD;

wherein said microorganism belongs to the genus *Escherichia* and has the ability to form a dipeptide from an L-amino acid amide and an L-amino acid by producing a protein having L-amino acid amide hydrolase activity, wherein said microorganism carries a recombinant DNA in which DNA encoding a protein having L-amino acid amide hydrolase activity is ligated to a vector DNA;

or

(B) a treated matter of the culture, wherein the treated matter of the culture is concentrated culture, dried culture, cells obtained by centrifuging the culture, or a product obtained by subjecting the cells to drying, freeze-drying, or immobilization;

- allowing the dipeptide to form and accumulate in the aqueous medium; and

- recovering the dipeptide from the medium.

9. The process of any one of claims 1, 3, 5 and 7, wherein the peptide-transporting protein is a protein having the amino acid sequence shown in any one of SEQ ID NOs: 5 to 9, or a protein having an amino acid sequence which has 80% or more identity to the full length of amino acid sequence shown in any one of SEQ ID NOs: 5 to 9 and having peptide-transporting activity.

10. The process of any one of claims 1 to 9, wherein the peptidase is a protein having the amino acid sequence shown in any one of SEQ ID NOs: 1 to 4, or a protein having an amino acid sequence which has 80% or more identity to the full length of amino acid sequence shown in any one of SEQ ID NOs: 1 to 4 and having peptidase activity.

Fig.1

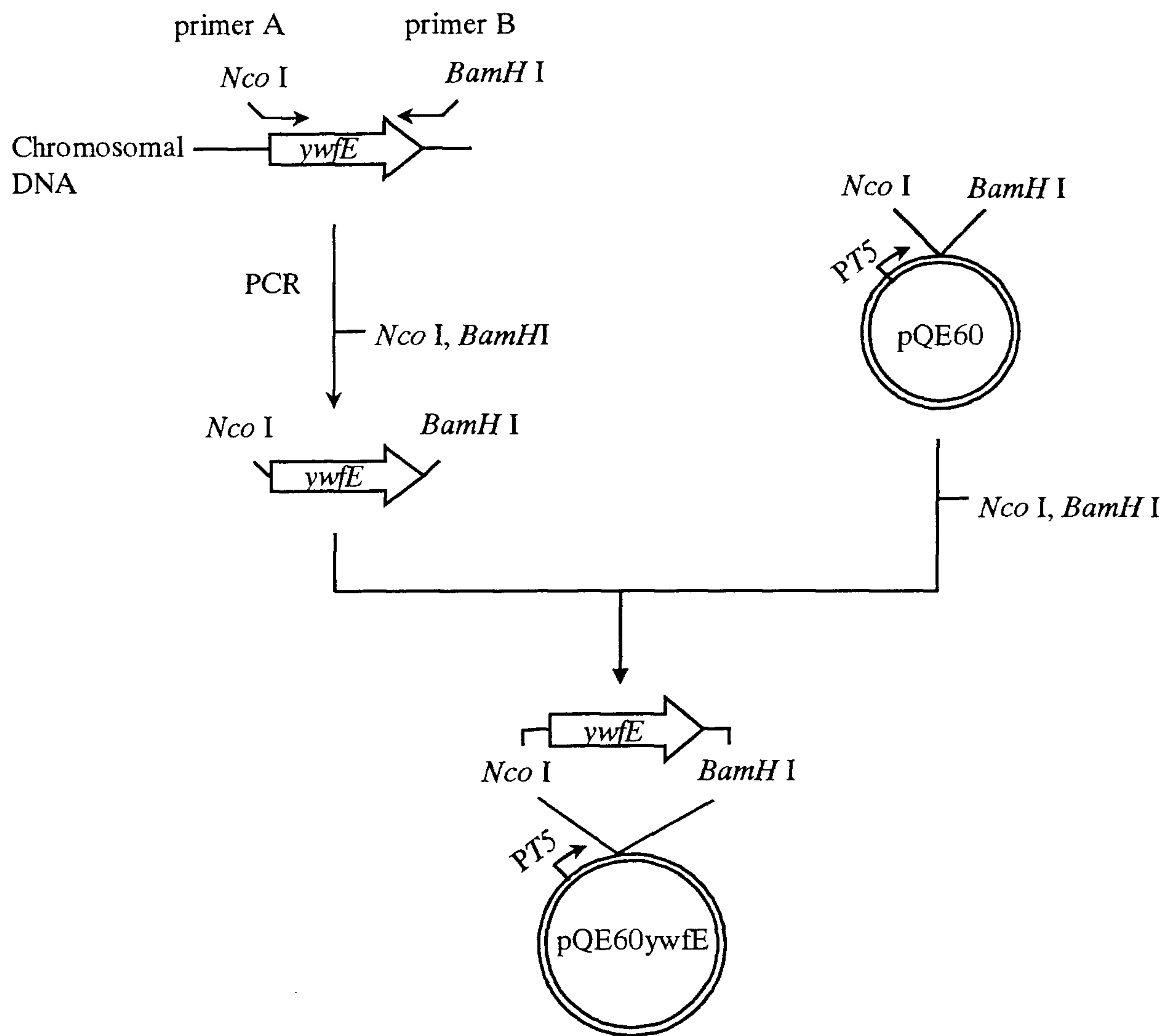


Fig. 2

