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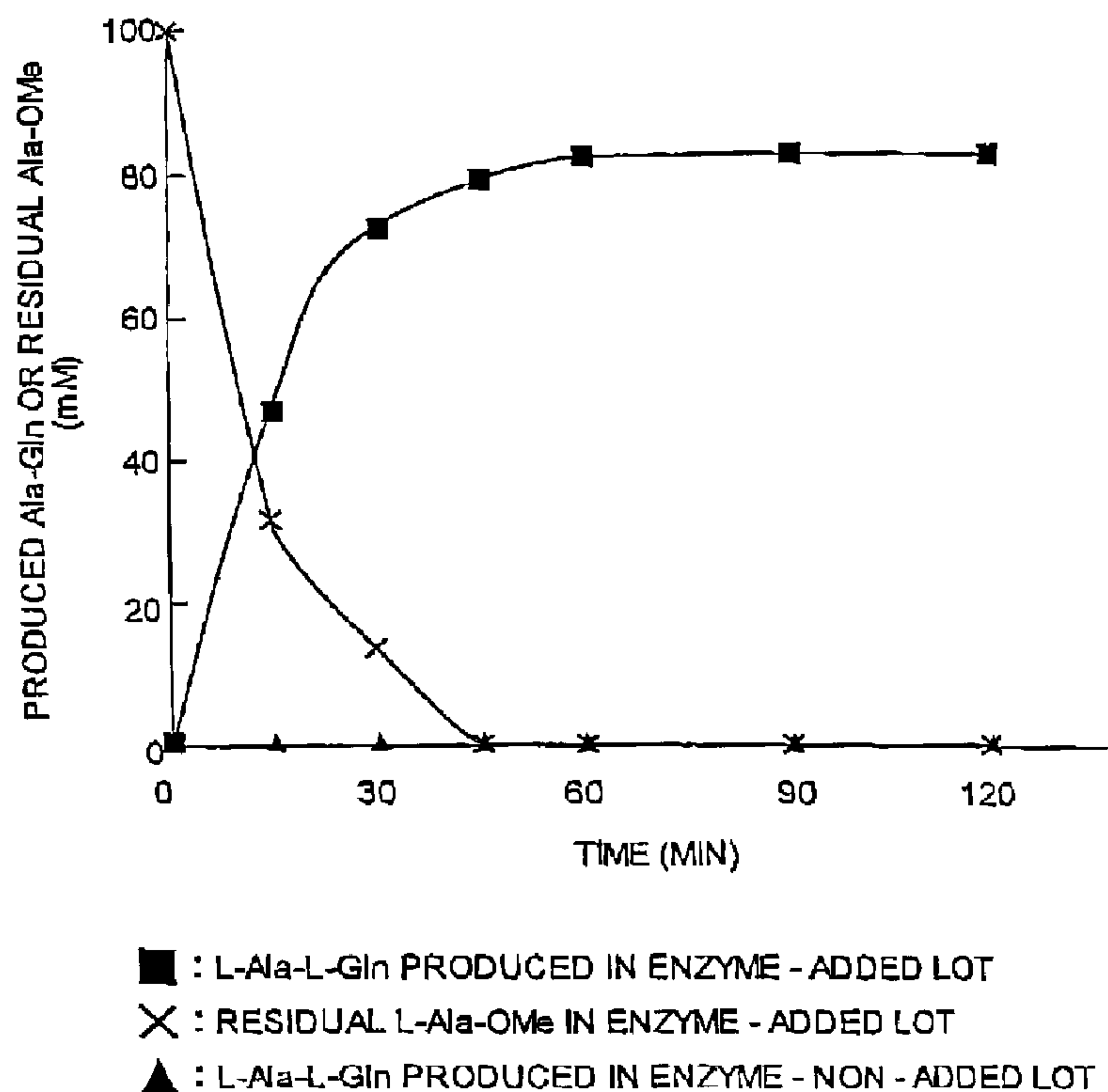
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(54) Titre : NOUVEAU PEPTIDE FORMANT UNE ENZYME, MICRO-ORGANISME PRODUISANT LEDIT PEPTIDE ET
PROCEDE DE PRODUCTION DUDIT PEPTIDE UTILISANT LESDITS ELEMENTS

(54) Title: NOVEL PEPTIDE-PRODUCING ENZYME, MICROBE PRODUCING THE ENZYME AND METHOD FOR
DIPEPTIDE SYNTHESIS USING THEM



(57) Abrégé/Abstract:

The present invention relates to a novel enzyme that allows peptide to be produced easily, inexpensively and at high yield without going through a complex synthesis method. More particularly, the present invention provides a novel enzyme that catalyzes a peptide-producing reaction from a carboxy component and an amine component, a microbe that produces the enzyme, and a method for inexpensive production of peptides using this enzyme or microbe. The novel enzyme that efficiently produces peptide was discovered from a newly discovered microbe belonging to the genus *Empedobacter*, and a method was found that allows peptides to be produced inexpensively and easily.

ABSTRACT

The present invention relates to a novel enzyme that allows peptide to be produced easily, inexpensively and at high yield without going through a complex synthesis method. More particularly, the present invention provides a novel enzyme that catalyzes a peptide-producing reaction from a carboxy component and an amine component, a microbe that produces the enzyme, and a method for inexpensive production of peptides using this enzyme or microbe. The novel enzyme that efficiently produces peptide was discovered from a newly discovered microbe belonging to the genus *Empedobacter*, and a method was found that allows peptides to be produced inexpensively and easily.

SPECIFICATION

TITLE OF THE INVENTION

Novel peptide-producing enzyme, microbe producing the
5 enzyme and method for dipeptide synthesis using them

TECHNICAL FIELD

The present invention relates to a novel enzyme that can
produce a peptide easily, inexpensively and at high yield without going
10 through a complex synthesis method. More particularly, the present
invention relates to a novel enzyme that catalyzes a peptide-producing
reaction from a carboxy component and an amine component, to a
microbe that produces the enzyme, and a method for producing a
dipeptide using the enzyme or microbe.

15

BACKGROUND ART

Peptides are used in the fields of pharmaceuticals, foods and
various other fields. For example, since L-alanyl-L-glutamine has
higher stability and water-solubility than L-glutamine, it is widely used
20 as a component of fluid infusion and serum-free media.

Chemical synthesis methods, which have been known as
methods for producing peptides, are not always easy. Known
examples of such methods include a method that uses
N-benzyloxycarbonylalanine (hereinafter, "Z-alanine") and protected
25 L-glutamine (see Bull. Chem. Soc. Jpn., 34, 739 (1961), Bull. Chem.

Soc. Jpn., 35, 1966 (1962)), a method that uses Z-alanine and protected L-glutamic acid- γ -methyl ester (see Bull. Chem. Soc. Jpn., 37, 200 (1964)), a method that uses Z-alanine ester and unprotected glutamic acid (see Japanese Patent Application Laid-open Publication
5 No. H1-96194), a method that involves synthesis of an N-(2-substituted)-propionyl glutamine derivative as an intermediate from a 2-substituted-propionyl halide as a raw material (see Patent Application Laid-open Publication No. H6-234715).

However, since all of these methods require the introduction and
10 elimination of protecting groups or the use of an optically active intermediate, they are not considered to be adequately satisfactory in terms of their industrial advantages.

On the other hand, widely known examples of typical peptide production methods using enzymes consist of a condensation reaction
15 that uses an N-protected and C-unprotected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 1"), and a substitution reaction that uses an N-protected, C-protected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 2"). An example of Reaction 1 is a
20 method for producing Z-aspartylphenylalanine methyl ester from Z-aspartic acid and phenylalanine methyl ester (see Japanese Patent Application Laid-open Publication No. S53-92729), while an example of Reaction 2 is a method for producing acetylphenylalanylleucine amide from acetylphenylalanine ethyl ester and leucine amide (see
25 Biochemical J., 163, 531 (1977)). There have been reported very few

research examples of methods that use an N-unprotected, C-protected carboxy component. An example of a substitution reaction that uses an N-unprotected, C-protected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 3") is described in International Patent Publication WO 90/01555. For example, a method for producing arginylleucine amide from arginine ethyl ester and leucine amide may be mentioned of. Examples of substitution reactions that use an N-unprotected, C-protected carboxy component and an N-unprotected, C-unprotected amine component (hereinafter, "Reaction 4") are described in European Patent Publications EP 278787A1 and EP 359399B1. For example, a method for producing tyrosylalanine from tyrosine ethyl ester and alanine may be mentioned of.

DISCLOSURE OF THE INVENTION

The most inexpensive production method among the aforementioned methods of Reactions 1 to 4 naturally falls within the class of Reaction 4, which involves the fewest protecting groups.

However, the example of Reaction 4 of the prior art (European Patent Publication EP 278787A1) had the following major problems:

- (1) extremely slow rate of peptide production,
- (2) low peptide production yield,
- (3) the peptides that can be produced are limited to those that contain amino acids with comparatively high hydrophobicity,
- (4) the amount of enzyme added is extremely large, and
- (5) comparatively expensive carboxypeptidase preparations derived

from molds, yeasts or plants are required. In the Reaction 4, there is no method known whatsoever that uses an enzyme derived from bacteria or yeasts other than the genus *Saccharomyces*, and there is no known method for producing alanylglutamine and other peptides that are highly hydrophilic.

5 In consideration of this background, there is a need to develop an industrially inexpensive method for producing these peptides.

It is an object of the present invention is to provide a novel enzyme that can form a peptide easily, inexpensively and at high yield without going through a complex synthesis method. More particularly, it is an object of the present invention to provide a novel enzyme that catalyzes a peptide-forming reaction from a carboxy component and an amine component, a microbe that produces the enzyme, and a method for inexpensively producing peptide using the enzyme or microbe.

The inventors of the present invention have found a novel enzyme that efficiently forms peptide from newly discovered bacteria belonging to the genus *Empedobacter* and so forth, and have completed the present invention.

The present invention is as described below:

[1] An enzyme derived from a microbe belonging to a genus selected from the genus *Empedobacter* and the genus *Sphingobacterium*, having the ability to form a peptide from a carboxy component and an amine component.

[2] An enzyme having the ability to form a peptide from a carboxy component and an amine component and the ability to form L-alanyl-L-glutamine at a formation rate of 0.03 mM/min or more in a dipeptide-forming reaction under the conditions (i) to (iv):

(i) the carboxy component is L-alanine methyl ester hydrochloride in an amount of 100 mM;

(ii) the amine component is L-glutamine in an amount of 200 mM;

(iii) the pH is 9.0; and,

5 (iv) the amount of homogeneously purified enzyme added is less than 0.61 mg/ml as protein.

[3] The enzyme according to [1] or [2], wherein the carboxy component as a substrate includes both the amino acid ester and the amino acid amide.

10 [4] The enzyme according to any one of [1] to [3], wherein any of an amino acid, a C-protected amino acid and an amine can be used as a substrate for the amine component.

[5] The enzyme according to any one of [1] to [4], wherein the enzyme has the ability to form a peptide within a pH range of 6.5 to 10.5.

15 [6] The enzyme according to any one of [1] to [5], wherein the enzyme has the ability to form a peptide within a temperature range of 0 to 60°C.

[7] The enzyme according to any one of [1] to [6], wherein the enzyme is not inhibited by a serine enzyme inhibitor, phenylmethanesulfonyl fluoride, but is inhibited by p-nitrophenyl-p'-guanidinobenzoate.

[8] The enzyme according to any one of [1] to [7], wherein the

enzyme has a molecular weight as determined by SDS-gel electrophoresis of about 75 kilodaltons, and a molecular weight as determined by gel filtration chromatography of about 150 kilodaltons.

[9] A microbe that produces an enzyme according to any one of [1] to [8].

[10] The microbe according to [9], wherein, the microbe is *Empedobacter brevis* strain FERM BP-8113 (Depository institution: National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of deposited institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) or *Sphingobacterium* sp. strain FERM BP-8124 (Depository institution: National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken,, Japan, International deposit transfer date: July 22, 2002).

[11] A method for producing a dipeptide, comprising producing a dipeptide from a carboxy component and an amine component using an enzyme according to any one of [1] to [8] or a substance containing the enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing an optimum pH of the enzyme of the present invention;

Fig. 2 is a diagram showing an optimum temperature of the

enzyme of the present invention; and

Fig. 3 is a diagram showing the time course in L-alanyl-L-glutamine production from L-alanine methyl ester and L-glutamine.

5

BEST MODE FOR CARRYING OUT THE INVENTION

Modes for carrying out the present invention are explained in detail in the order of

- (1) Microbe Producing the Enzyme of the Present Invention,
- 10 (2) Microbe Culturing,
- (3) Enzyme Purification,
- (4) Enzyme Properties, and
- (5) Dipeptide Synthesis Method.

- 15 (1) Microbe Producing the Enzyme of the Present Invention

The enzyme of the present invention may be any enzyme that has the ability to produce a peptide from a carboxy component and an amine component, and there are no particular restriction on organisms that produce such an enzyme. In the present specification, the

20 carboxy component refers to the component that provides a carbonyl site (CO) in the peptide bond (-CONH-), while the amine component refers to the component that provides the amino site (NH) in the peptide bond. In addition, in the present specification, the term "peptide" used alone refers to a polymer having at least one or more peptide

25 bonds unless otherwise indicated specifically. In addition, the term

"dipeptide" in the present specification refers to a peptide having one peptide bond.

Examples of microbes that produce the enzyme of the present invention include bacteria belonging to the genus *Empedobacter* and so forth, specific examples of which include *Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545), and *Sphingobacterium* sp. strain FERM BP-8124. *Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545, strain FERM BP-8113) and *Sphingobacterium* sp. strain FERM BP-8124 are microbes that were screened by the inventors of the present invention as a result of searching for microbes that produce a peptide from a carboxy component and an amine component at high yield. Microbes having similar bacteriological properties to those of *Empedobacter brevis* strain ATCC 14234 (strain FERM P-18545) or *Sphingobacterium* sp. strain FERM BP-8124 are also microbes that produce the enzyme of the present invention.

Empedobacter brevis strain ATCC 14234 (strain FERM P-18545) was deposited at the International Patent Organism Depository of the National Institute for Advanced Industrial Science and Technology (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on October 1, 2001 and assigned the deposit number of FERM P-18545. Control of this organism was subsequently transferred to deposition under the provisions of the Budapest Treaty at the International Patent Organism Depository of the National Institute for Advanced Industrial Science and Technology on July 8, 2002 and was assigned the deposit number of FERM BP-8113 (indication of

microbe: *Empedobacter brevis* strain AJ 13933).

Sphingobacterium sp. strain AJ 110003 was deposited at the International Patent Organism Depositary of the National Institute for Advanced Industrial Science and Technology on July 22, 2002, and was
5 assigned the deposit number of FERM BP-8124. It should be noted that strain AJ 110003 was identified to be the aforementioned *Sphingobacterium* sp. by the identification experimentation described below. Strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology,
10 International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken,, Japan, International deposit date: July 22, 2002) is a Gram-negative rod (0.7 to 0.8 × 1.5 to 2.0 micrometers (μm)) that does not form spores and is not motile. Its colonies are round with a completely smooth border, contain low protrusions
15 and have a glossy, light yellow color. The organism grows at 30°C and is catalase positive, oxidase positive and negative for the OF test (glucose), and was identified as a bacterium belonging to the genus *Sphingobacterium* based on these properties. Moreover, from the properties of being negative for nitrate reduction, negative for indole production, negative for acid
20 production from glucose, arginine dihydrolase negative, urease positive, esculin hydrolysis positive, gelatin hydrolysis negative, β-galactosidase positive, glucose assimilation positive, L-arabinose assimilation negative, D-mannose assimilation positive, D-mannitol assimilation negative, N-acetyl-D-glucosamine assimilation positive, maltose assimilation

positive, potassium gluconate assimilation negative, n-capric acid
negative, adipic acid assimilation negative, dl-malic acid assimilation
negative, sodium citrate assimilation negative, phenyl acetate
assimilation negative and cytochrome oxidase positive, the microbe was
5 determined to have properties that are similar to those of
Sphingobacterium multivorum or *Sphingobacterium spiritivorum*.
Moreover, as a result of analyzing homology of the base sequence of
16S rRNA gene, although the highest degree of homology was exhibited
with *Sphingobacterium multivorum* (98.8%), there was no strain with
10 which it matched completely. This bacterial strain was therefore
identified as *Sphingobacterium sp.*

The enzyme of the present invention can be obtained by
isolating and purifying from the cells of the above-mentioned
Empedobacter brevis or *Sphingobacterium sp.* In addition, the enzyme
15 of the present invention as well as microbes that produce the enzyme
can also be obtained by genetic engineering techniques based on the
isolated enzyme. Namely, the enzyme and microbe of the present
invention can be produced by isolating a DNA that encodes the enzyme
of the present invention based on the isolated and purified enzyme
20 followed by expressing the DNA by introducing the DNA into a suitable
host. In addition, an enzyme having the ability to produce a peptide
from a carboxy component and an amine component can also be
obtained from other microbes by producing a probe based on a
polynucleotide and so forth that encodes the enzyme of the present
25 invention obtained from *Empedobacter brevis*. Various gene

recombination techniques are described in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989) and other publications.

5 (2) Microbe Culturing

In order to obtain cultured cells of microbes having the enzyme used in the present invention, it suffices that the microbes be cultured and grown in a suitable medium. There is no particular restriction on the medium used for this purpose so far as it allows the microbes to grow. This medium may
10 be an ordinary medium containing ordinary carbon sources, nitrogen sources, phosphorus sources, sulfur sources, inorganic ions, and organic nutrient sources as necessary.

For example, any carbon source may be used so far as the microbes can utilize it. Specific examples of the carbon source that can be used
15 include sugars such as glucose, fructose, maltose and amylose, alcohols such as sorbitol, ethanol and glycerol, organic acids such as fumaric acid, citric acid, acetic acid and propionic acid and their salts, hydrocarbons such as paraffin as well as mixtures thereof.

Examples of nitrogen sources that can be used include ammonium
20 salts of inorganic salts such as ammonium sulfate and ammonium chloride, ammonium salts of organic acids such as ammonium fumarate and ammonium citrate, nitrates such as sodium nitrate and potassium nitrate, organic nitrogen compounds such as peptones, yeast extract, meat extract and corn steep liquor as well as mixtures thereof.

In addition, ordinary nutrient sources used in media, such as

inorganic salts, trace metal salts and vitamins, can also be suitably mixed and used.

There are no particular restrictions on culturing conditions, and culturing can be carried out, for example, for about 12 to about 48
5 hours while properly controlling the pH and temperature to a pH range of 5 to 8 and a temperature range of 15 to 40°C, respectively, under aerobic conditions.

(3) Enzyme Purification

A method for isolating and purifying a peptide-producing enzyme
10 from *Empedobacter brevis* is explained as an example of purifying the enzyme of the present invention. First, a microbial cell extract is prepared from the cells of, for example, *Empedobacter brevis* strain FERM BP-8113 (Depository institution: National Institute for Advanced Industrial Science and Technology, International Patent Organism
15 Depository, Address of deposited institution: Central 6, 1-1-1 Higashi, Tsukuba City, Ibaraki Prefecture, Japan, International deposit transfer date: July 8, 2002) by disrupting the cells using a physical method such as ultrasonic crushing or an enzymatic method using a cell wall-dissolving enzyme and removing the insoluble fraction by
20 centrifugal separation and so forth.

A peptide-producing enzyme can then be purified from the cell extract obtained in the above manner by combining ordinary protein purification methods such as anion exchange chromatography, cation exchange chromatography or gel filtration chromatography.

25 An example of a carrier for use in anion exchange

chromatography is Q-Sepharose* HP (manufactured by Amersham).

The enzyme is recovered in the non-adsorbed fraction under conditions of pH 8.5 when the cell extract containing the enzyme is allowed to pass through a column packed with the carrier.

5 An example of a carrier for use in cation exchange chromatography is MonoS HR (manufactured by Amersham). After adsorbing the enzyme onto the carrier (in the column) by allowing the cell extract containing the enzyme to pass through a column packed with the carrier and then washing the column, the enzyme is eluted with
10 a buffer solution having a high salt concentration. At that time, the salt concentration may be sequentially increased or gradiently increased. For example, in the case of using MonoS HR, the enzyme adsorbed onto the carrier is eluted at an NaCl concentration of about 0.2 to about 0.5 M.

15 The enzyme purified in the manner described above can then be further homogeneously purified by gel filtration chromatography and so forth. An example of the carrier for use in gel filtration chromatography is Sephadex* 200pg (manufactured by Amersham).

 The fraction that contains the present enzyme in the
20 aforementioned purification procedure can be confirmed by assaying the peptide production activity of each fraction according to the method described later.

(4) Properties of Enzyme of the Present Invention

 While the enzyme of the present invention is an enzyme that
25 has the ability to produce a peptide from a carboxy component and an

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amine component,

a preferable mode of the enzyme of the present invention will be explained hereinbelow from the standpoint of its properties.

5 An enzyme having the abilities described below, for which the dipeptide formation rate is used as an indicator, is one preferable mode of the enzyme of the present invention. Namely, a preferable mode of the enzyme of the present invention is an enzyme that has the ability to form a peptide from a carboxy component and an amine component, and the ability to exhibit
10 a formation rate of L-alanyl-L-glutamine of preferably 0.03 mM/min or more, more preferably 0.3 mM/min or more, and particularly preferably 1.0 mM/min or more in the dipeptide-forming reaction under the conditions of (i) to (iv) below. The conditions of the dipeptide-forming reaction are as follows:

(i) The carboxy component is L-alanine methyl ester hydrochloride (100
15 millimolar (mM));

(ii) The amine component is L-glutamine (200 mM);

(iii) The pH is 9.0; and,

(iv) The amount of homogeneously purified enzyme added is less than 0.61 mg/ml as protein amount.

20 The aforementioned amount of enzyme added indicates a final amount of added enzyme that is added to the reaction system, and addition of the enzyme of 0.01 mg/ml or more, and preferably 0.02 mg/ml or more, as protein amount is desirable. The term "protein amount" refers to the value indicated with the Coomassie brilliant blue colorimetric method using protein

assay CBB solution (manufactured by Nakarai) and bovine serum albumin for the standard substance.

The aforementioned formation rate far exceeds the conventional formation rate for peptide formation using an enzyme, and the enzyme of the present invention has the ability to catalyze peptide formation at an extremely high rate.

As a specific example of a procedure for assaying enzyme activity, enzyme activity can be assayed by allowing the enzyme to react in a borate buffer solution containing an amino acid ester and an amine as substrates followed by quantifying the resulting peptide. As a more specific example, the enzyme is allowed to react for several minutes at 25°C using a 100 mM borate buffer solution (pH 9.0) containing 100 mM L-alanine methyl ester and 200 mM L-glutamine.

The enzyme activity unit used in the present invention is defined such that 1 unit (U) is the amount of enzyme that forms 1 micromole of peptide in 1 minute under the condition of reacting at 25°C using 100 mM borate buffer solution (pH 9.0) containing 100 mM L-alanine methyl ester and 200 mM L-glutamine.

In addition, a preferable mode of the enzyme of the present invention is an enzyme having the property by which both an amino acid ester and an amino acid amide can be used as a substrate for the carboxy component. The words "both an amino acid ester and an amino acid amide can be used as a substrate" mean that at least one type of amino acid ester and at least one type of amino acid amide can be used as a substrate. In addition, one

preferable mode of the enzyme of the present invention is an enzyme that has the property by which any of an amino acid, a C-protected amino acid and an amine can be used as a substrate for the amine component. The words "an amino acid, a C-protected amino acid, and an amine can be used
5 as a substrate" mean that at least one type of amino acid, at least one type of C-protected amino acid and at least one type of amine can be used as a substrate. As a result of having a wide range of substrate specificity with respect to the carboxy component or the amino component, the enzyme of the present invention is preferable in the sense that a wide range of raw
10 materials can be selected, which in turn is favorable in terms of cost and production equipment in the case of industrial production.

Specific examples of carboxy components include L-amino acid esters, D-amino acid esters, L-amino acid amides and D-amino acid amides. In addition, amino acid esters include not only amino acid esters corresponding
15 to naturally-occurring amino acids, but also amino acid esters corresponding to non-naturally-occurring amino acids or their derivatives. Further, examples of amino acid esters include α -amino acid esters as well as β -, γ -, and ω -amino acid esters and the like, which have different amino group bonding sites. Typical examples of amino acid esters include methyl esters,
20 ethyl esters, n-propyl esters, iso-propyl esters, n-butyl esters, iso-butyl esters and tert-butyl esters of amino acids, etc.

Specific examples of the amine components include L-amino acids, C-protected L-amino acids, D-amino acids, C-protected D-amino

acids and amines. In addition, examples of the amines include not only naturally-occurring amines, but also non-naturally-occurring amines or their derivatives. In addition, examples of the amino acids include not only naturally-occurring amino acids, but also

5 non-naturally-occurring amino acids or their derivatives. These include α -amino acids as well as β -, γ - or ω -amino acids, which have different amino group bonding sites.

In addition, in a different aspect, one preferable mode of the enzyme of the present invention is an enzyme in which the pH range
10 over which the peptide-producing reaction can be catalyzed is 6.5 to 10.5. The ability of the enzyme of the present invention to catalyze this reaction over a wide pH range is preferable in that it allows flexible accommodation of industrial production that could be subject to the occurrence of various restrictions. However, in the actual production
15 of peptide, it is preferable to use the enzyme by further adjusting to an optimum pH corresponding to the acquired enzyme so as to maximize the catalytic performance of the enzyme.

Moreover, an example of another different aspect of a preferable mode of the enzyme of the present invention is an enzyme for which the
20 temperature range over which the enzyme is capable of catalyzing the peptide-producing reaction is within the range of 0 to 60°C. Since the enzyme of the present invention is able to catalyze the reaction over a wide temperature range, it is preferable in that it allows flexible accommodation of industrial production that could be subject to the
25 occurrence of various restrictions. However, in the actual production

of

peptides, it is preferable to use the enzyme by further adjusting to an optimum temperature corresponding to the acquired enzyme so as to maximize the catalytic performance of the enzyme.

5 (5) Dipeptide Synthesis Method

The method for producing a dipeptide according to the present invention comprises synthesizing a dipeptide by allowing an enzyme having the ability to form a peptide from a carboxy component and an amine component or a substance that contains that enzyme to act on the carboxy
10 component and the amine component.

For the method for allowing the enzyme used in the present invention or enzyme-containing substance to act on the carboxy component and the amine component, it suffices that the enzyme or enzyme-containing substance, carboxy component and amine component be mixed. More
15 specifically, a method may be used in which an enzyme or enzyme-containing substance is added to a solution containing the carboxy component and the amine component and allowed to react, or in the case of using a microbe that produces the enzyme, a method may be used in which the microbe that produces the enzyme is cultured, the enzyme present in the microbe or
20 microbial culture broth is produced and accumulated, and the carboxy component and amine component are then added to the culture broth. The formed dipeptide can then be collected by established methods and purified as necessary.

The term "enzyme-containing substance" refers to that which contains the enzyme, examples of specific forms thereof include a

culture of microbes that produce the enzyme, microbial cells isolated from the culture, and a treated microbial cell product. A culture of microbes refers to that which is obtained by culturing microbes, and more specifically, to a mixture of microbial cells, medium used for
5 culturing the microbes, and substances produced by the cultured microbes and the like. In addition, the microbial cells may be washed and used in the form of washed microbial cells. Further, a treated microbial cell product includes the crushed, lysed or freeze-dried microbial cells, and also includes a crude enzyme recovered by
10 processing microbial cells and so forth as well as a purified enzyme obtained by purification of the crude enzyme. A partially purified enzyme obtained by various types of purification methods may be used for the purified enzyme, or immobilized enzyme may be used that has been immobilized by covalent bonding, adsorption or entrapment
15 methods. In addition, since some microbes are partially lysed during culturing depending on the microbes used, the culture supernatant may also be used as the enzyme-containing substance in such cases.

In addition, not only wild strains but also genetic recombinant strains may be used for the microbes that contain the enzyme. The
20 microbes are not limited to intact cells, but rather acetone-treated microbial cells, freeze-dried microbial cells or other treated microbial cells may also be used. Immobilized microbial cells immobilized by covalent bonding, adsorption, entrapment or other methods, as well as treated immobilized microbial cells, may also be used.

25 It should be noted that in the case of using cultures, cultured

microbial cells or a treated microbial cell product, there are many cases in which an enzyme exists that does not participate in production of peptides but decomposes the produced peptides, and in such cases, it is rather preferable to add a metal protease inhibitor like

5 ethylenediaminetetraacetic acid (EDTA) depending on the cases. The addition amount is within the range of 0.1 mM to 300 mM, and preferably 1 mM to 100 mM.

The amount of enzyme or enzyme-containing substance used should be an amount at which the target effect is demonstrated

10 (hereinafter, "effective amount"). Although this effective amount can be easily determined through simple, preliminary experimentation by a person with ordinary skill in the art, in the case of using an enzyme, for example, the use amount thereof is about 0.01 to 100 units (U), while in the case of using washed microbial cells, the use amount thereof is

15 about 1 to 500 g/L.

Any carboxy component may be used provided that it is capable of producing a peptide by condensation with the other substrate in the form of the amine component. Examples of the carboxy components include L-amino acid esters, D-amino acid esters, L-amino acid amides

20 and D-amino acid amides. Examples of the amino acid esters include not only amino acid esters corresponding to naturally-occurring amino acids, but also amino acid esters corresponding to non-naturally-occurring amino acids or their derivatives. In addition, examples of the amino acid esters include α -amino acid esters as well

25 as β -, γ - and ω -amino acid esters and the like, which having different

amino group bonding sites. Typical examples of the amino acid esters include methyl esters, ethyl esters, n-propyl esters, iso-propyl esters, n-butyl esters, iso-butyl esters and tert-butyl esters of amino acids.

Any amine component may be used provided that it is capable
5 of producing peptide by condensation with the other substrate in the form of the carboxy component. Examples of the amine components include L-amino acids, C-protected L-amino acids, D-amino acids, C-protected D-amino acids and amines. In addition, examples of the amines include not only naturally-occurring amines, but also
10 non-naturally-occurring amines or their derivatives. Examples of the amino acids include not only naturally-occurring amino acids, but also non-naturally-occurring amino acids or their derivatives. These include α -amino acids as well as β -, γ - or ω -amino acids, which have different amino group bonding sites.

15 Although the concentrations of the carboxy component and amine component serving as starting materials are 1 mM to 10 M, and preferably 0.05 mole (hereinafter, "M") to 2 M, respectively, there are cases in which it is preferable to add the amine component in an amount equal to or greater than that of the carboxy component. In
20 addition, in the case of substrates that inhibit the reaction at high concentrations, these can be adjusted to a concentration that does not result in inhibition and successively added during the reaction.

The reaction temperature that allows production of a peptide is 0 to 60°C, and preferably 5 to 40°C. The reaction pH that allows
25 production of a peptide is 6.5 to 10.5, and preferably 7.0 to 10.0.

Examples

Although the following provides a more detailed explanation of the present invention through its examples, the present invention is not limited to these examples. In addition to confirmation by ninhydrin coloring of thin-film chromatograms (qualitative), quantitative determinations were made by the following high-performance liquid chromatography in order to assay products. Column: Inertsil ODS-2 (manufactured by GL Science, Inc.), eluate: aqueous phosphate solution containing 5.0 mM sodium 1-octanesulfonate (pH 2.1) : methanol = 100 : 15 to 50, flow rate: 1.0 mL/min, detection: 210 nanometers (nm).

Example 1 Microbe Culturing (*Empedobacter brevis* Strain FERM BP-8113)

A 50 mL medium (pH 6.2) containing 5 grams (g) of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 g of peptone in 1 liter (L) was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes. This medium was then inoculated with one loopful of the culture broth of *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism

Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi
1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit
transfer date: July 8, 2002) that had been cultured at 30°C for 16 hours
in the same medium, followed by shake culturing at 30°C for 16 hours
5 and 120 strokes/min.

Example 2 Production of Peptide Using Microbial Cells

Microbial cells were collected by centrifuging (10,000 rounds per
minute (rpm), 15 minutes) the culture broth obtained in Example 1
10 followed by suspending to a concentration of 100 g/L in 100 mM borate
buffer (pH 9.0) containing 10 mM EDTA. After respectively adding 1
mL of this suspension to 1 mL of 100 mM borate buffer (pH 9.0)
containing 10 mM EDTA, 200 mM of the following carboxy component
and 400 mM of the following amino acids to bring to a final volume of 2
15 mL, the reaction was carried out at 18°C for 2 hours. The peptides
that were formed as a result of this reaction are shown in Table 1.

Table 1

Carboxy com- ponent	Amine com- ponent	Produced peptide	(mM)	Carboxy com- ponent	Amine com- ponent	Produced peptide	(mM)
L-Ala-OMe	L-Leu	L-Ala-L-Leu	38.2	Gly-OMe	L-His	L-Gly-L-His	22.1
	L-Met	L-Ala-L-Met	68.3	L-Ser-OMe	L-Ser	L-Ser-L-Ser	29.0
	L-Phe	L-Ala-L-Phe	62.4	L-Val-OMe	L-Met	L-Val-L-Met	10.5
	L-Ser	L-Ala-L-Ser	51.3	L-Met-OMe	L-Phe	L-Met-L-Phe	28.5
	L-His	L-Ala-L-His	52.1	L-Thr-OMe	L-Leu	L-Thr-L-Leu	23.0
	L-Arg	L-Ala-L-Arg	72.1	L-Ile-OMe	L-Met	L-Ile-L-Met	8.3
	L-Gln	L-Ala-L-Gln	68.0				

Hydrochloride salts were used for all the carboxy components.

Example 3 Enzyme Purification

- 5 The procedure after centrifugal separation was carried out either on ice or at 4°C. *Empedobacter brevis* strain FERM BP-8113 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository
- 10 institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken,, Japan, International deposit transfer date: July 8, 2002) was cultured in the same manner in as Example 1, and the microbial cells were collected by centrifugal separation (10,000 rpm, 15 minutes). After washing 16 g of microbial cells with 50 mM Tris-HCl buffer (pH 8.0),
- 15 they were suspended in 40 milliliters (ml or mL) of the same buffer and subjected to ultrasonic crushing

treatment for 45 minutes at 195 watts. This ultrasonic crushing liquid was then centrifuged (10,000 rpm, 30 minutes) to remove the crushed cell fragments and obtain an ultrasonic crushing liquid supernatant. This ultrasonic crushing liquid supernatant was dialyzed overnight
5 against 50 mM Tris-HCl buffer (pH 8.0) followed by removal of the insoluble fraction by ultracentrifugation (50,000 rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant liquid. The resulting soluble fraction was applied to a Q-Sepharose HP column (manufactured by Amersham) pre-equilibrated with Tris-HCl buffer (pH
10 8.0), and the active fraction was collected from the non-adsorbed fraction. This active fraction was dialyzed overnight against 50 mM acetate buffer (pH 4.5) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the supernatant liquid. This dialyzed fraction
15 was then applied to a Mono S column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) to elute enzyme at a linear concentration gradient of the same buffer containing 0 to 1 M NaCl. The fraction that had the lowest level of contaminating protein among the active fractions was applied to a Superdex 200pg column
20 (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) containing 1 M NaCl, and gel filtration was performed by allowing the same buffer (pH 4.5) containing 1 M NaCl to flow through the column to obtain an active fraction solution. As a result of performing these procedures, the peptide-producing enzyme used in
25 the present invention was confirmed to have been uniformly purified

based on the experimental results of electrophoresis. The enzyme recovery rate in the aforementioned purification process was 12.2% and the degree of purification was 707 times.

5

Example 4 Measurement of Enzyme Molecular Weight

SDS-Gel Electrophoresis

A 0.3 microgram (μg) of the purified enzyme fraction obtained by the method of Example 3 was applied to polyacrylamide electrophoresis. 0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) sodium laurylsulfate were used for the electrophoresis buffer solution, a gel having a concentration gradient of a gel concentration of 10 to 20% (Multigel*10 to 20, manufactured by Daiichi Pure Chemicals) was used for the polyacrylamide gel, and Pharmacia molecular weight markers were used for the molecular weight markers. Following completion of electrophoresis, the gel was stained with Coomassie brilliant blue R-250, and a uniform band was detected at the location of a molecular weight of about 75 kilodalton (kDa).

15

Gel filtration

The purified enzyme fraction obtained by the method of Example 3 was applied to a Superdex 200pg column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) containing 1 M NaCl, and gel filtration was carried out by allowing the same buffer (pH 4.5) containing 1 M NaCl to flow through the column to measure the molecular weight. Pharmacia molecular weight markers

25

were used as standard proteins having known molecular weights to prepare a calibration curve. As a result, the molecular weight of the enzyme was about 150 kDa.

Based on the results of SDS-gel electrophoresis and gel
5 filtration, the enzyme was suggested to be a homodimer having a molecular weight of about 75 kDa.

Example 5 Enzyme Optimum pH

The effects of pH were examined in the reaction in which
10 L-alanyl-L-glutamine is formed from L-alanine methyl ester hydrochloride and L-glutamine. Acetate buffer (pH 3.9 to 5.4), MES buffer (pH 5.4 to 6.4), phosphate buffer (pH 6.0 to 7.9), borate buffer (pH 7.8 to 9.3), CAPS buffer (pH 9.3 to 10.7) and K₂HPO₄-NaOH buffer (pH 10.8 to 11.6) were used as buffers. 1 microliter (μl) of the Mono S
15 fraction enzyme obtained in Example 3 (about 180 U/ml) was added to 100 μl of each buffer (100 mM) containing 100 mM L-alanine methyl ester, 200 mM L-glutamine and 10 mM EDTA and allowed to react at 18°C for 5 minutes to measure the effects of pH on the reaction. The results based on assigning a value of 100% to the case of using borate
20 buffer (pH 9.3) are shown in Fig. 1. As a result, the optimum enzyme pH was 8 to 9.5.

Example 6 Enzyme Optimum Temperature

The effects of temperature were examined on the reaction in
25 which L-alanyl-L-glutamine is formed from L-alanine methyl ester

hydrochloride and L-glutamine. A 1 μ l aliquot of the same enzyme fraction used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester, 200 mM L-glutamine and 10 mM EDTA and allowed to react for 5 minutes at each temperature to measure the effects of temperature on the reaction. The results based on assigning a value of 100% to the activity at 34°C are shown in Fig. 2. As a result, the optimum enzyme temperature was 30 to 40°C.

10 Example 7 Enzyme Inhibitors

The effects of inhibitors on the production of L-alanyl-L-glutamine were examined using L-alanine methyl ester hydrochloride and L-glutamine as substrates. A 2 μ l aliquot of the same enzyme fraction used in Example 5 was added to 50 μ l of 100 mM borate buffer (pH 9.0) containing each of the enzyme inhibitors shown in Table 2 at 10 mM, and allowed to react at 25°C for 5 minutes. Note that, o-phenanthroline, phenylmethylsulfonyl fluoride and p-nitrophenyl-p'-guanidinobenzoate were dissolved in methanol to a concentration of 50 mM before use. The enzyme activity under each condition was indicated as the relative activity in the case of assigning a value of 100 to the production of L-alanyl-L-glutamine in the absence of enzyme inhibitor. Those results are shown in Table 2. As a result, among the serine enzyme inhibitors tested, the enzyme was not inhibited by phenylmethylsulfonyl fluoride, but it was inhibited by p-nitrophenyl-p'-guanidinobenzoate.

Table 2

Enzyme inhibitor		Relative activity of L-Ala-L-Gln production (%)
None		100
Metal enzyme inhibitor	EDTA	96
	o-Phenanthroline	96
SH enzyme inhibitor	N-Ethyl maleimide	110
	Monoiodoacetate	101
Serine enzyme inhibitor	Phenylmethylsulfonyl fluoride	115
	4-(2-Aminoethyl)benzene sulfonyl fluoride	75
	p-Nitrophenyl-p'-guanidino benzoate	0.1

Example 8 Production of L-Alanyl-L-Glutamine from L-Alanine Methyl Ester and L-Glutamine

- 5 A 3 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, 200 mM L-glutamine and 10 mM EDTA, and allowed to react at 18°C. As a result, as shown in Fig. 3, 83 mM L-alanyl-L-glutamine (L-Ala-L-Gln) was formed in the case of
- 10 an enzyme-added lot, and the concentration of by-product L-Ala-L-Ala-L-Gln was 1.3 mM. On the other hand, there was hardly any production of L-Ala-L-Gln observed in an enzyme-non-added lot, and the enzyme concentration was only about 0.07 mM after reacting for 120 minutes.

Example 9 Effects of L-Glutamine Concentration on Production of L-Alanyl-L-Glutamine

A 1 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, L-glutamine at the concentrations shown in Table 3 and 10 mM EDTA, and allowed to react at 18°C for 2 hours. Those results are shown in Table 3.

Table 3

L-Alanine methyl ester hydrochloride (mM)	L-Glutamine (mM)	L-Ala-L-Gln (mM)
100	100	68.2
	110	72.1
	120	73.3
	130	75.1
	150	75.5
	200	82.0

10

Example 10 Enzyme Substrate Specificity (1)

Ester specificity was examined in the case of using L-amino acid ester for the carboxy component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the carboxy components indicated in Table 4 at 100 mM, 200 mM L-glutamine and 10 mM EDTA, and allowed to react at 25°C for 2 hours. The amounts of L-Ala-L-Gln formed in this reaction are shown in Table 4 (HCl represents hydrochloride in Table 4).

Table 4

Carboxy component	L-Ala-L-Gln produced (mM)
L-Alanine methyl ester·HCl	84.3
L-Alanine ethyl ester·HCl	91.5
L-Alanine isopropyl ester·HCl	78.9
L-Alanine-t-butyl ester·HCl	7.5

Example 11 Enzyme Substrate Specificity (2)

Peptide production was examined in the case of using L-alanine methyl ester for the carboxy component and using various L-amino acids for the amine component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, the L-amino acids shown in Table 5 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 5. {Note that
~~The~~the “+” mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard, while “tr” indicates a trace amount.}

Table 5

Amine component	Produced peptide (mM)		Amine component	Produced peptide (mM)	
Gly	L-Ala-Gly	13.7	L-Asn	L-Ala-L-Asn	65.5
L-Ala	L-Ala-L-Ala	25.4	L-Gln	L-Ala-L-Gln	79.3
L-Val	L-Ala-L-Val	20.8	L-Tyr	L-Ala-L-Tyr	17.6
L-Leu	L-Ala-L-Leu	45.3	L-CySH	L-Ala-L-CySH	+
L-Ile	L-Ala-L-Ile	33.9	L-Lys	L-Ala-L-Lys	71.8
L-Met	L-Ala-L-Met	83.3	L-Arg	L-Ala-L-Arg	88.0
L-Phe	L-Ala-L-Phe	74.4	L-His	L-Ala-L-His	66.9
L-Trp	L-Ala-L-Trp	53.9	L-Asp	L-Ala-L-Asp	2.1
L-Ser	L-Ala-L-Ser	62.5	L-Glu	L-Ala-L-Glu	42.9
L-Thr	L-Ala-L-Thr	53.9	L-Pro	L-Ala-L-Pro	tr

Example 12 Enzyme Substrate Specificity (3)

Peptide production was examined in the case of using various types of L-amino acid methyl esters for the carboxy component and using L-glutamine for the amine component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the L-amino acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 6 at 100 mM, 150 mM L-glutamine and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 6. Note that the "+" mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount.)

Furthermore, Tween^{*}-80 was added to the reaction system to a final concentration of 0.1% in the case of using L-Trp-OMe and L-Tyr-OMe.

*Trade-mark

Table 6

Carboxy component	Produced peptide (mM)		Carboxy component	Produced peptide (mM)	
Gly-OMe	Gly-L-Gln	54.7	L-Tyr-OMe	L-Tyr-L-Gln	3.4
L-Ala-OMe	L-Ala-L-Gln	74.6	CySH-OMe	L-CySH-L-Gln	+
L-Val-OMe	L-Val-L-Gln	15.4	L-Lys-OMe	L-Lys-L-Gln	+
L-Leu-OMe	L-Leu-L-Gln	+	L-Arg-OMe	L-Arg-L-Gln	+
L-Ile-OMe	L-Ile-L-Gln	8.4	L-His-OMe	L-His-L-Gln	7.1
L-Met-OMe	L-Met-L-Gln	12.0	L-Asp- α -OMe	α -L-Asp-L-Gln	+
L-Phe-OMe	L-Phe-L-Gln	0.9	L-Asp- β -OMe	β -L-Asp-L-Gln	tr
L-Trp-OMe	L-Trp-L-Gln	+	L-Glu- α -OMe	α -L-Glu-L-Gln	tr
L-Ser-OMe	L-Ser-L-Gln	+	L-Glu- γ -OMe	γ -L-Glu-L-Gln	+
L-Thr-OMe	L-Thr-L-Gln	24.0	L-Pro-OMe	L-Pro-L-Gln	+
L-Asn-OMe	L-Asn-L-Gln	81.9			+
L-Gln-OMe	L-Gln-L-Gln	+			2.2
		0.3			

Hydrochloride salts were used for all of the carboxy components.

Example 13 Enzyme Substrate Specificity (4)

- 5 Peptide production was examined in the case of using various L-amino acid methyl esters for the carboxy component and various L-amino acids for the amine component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the L-amino acid methyl ester
- 10 hydrochlorides (AA-OMe·HCl) shown in Table 7 at 100 mM, the L-amino acids shown in Table 7 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts formed of each of the peptides formed in this reaction are shown in Table 7. Note that the “tr” indicates a trace amount. Furthermore, Tween-80 was added to the
- 15 reaction system to a final concentration of 0.1% in the case of using L-Trp-OMe. Note that the “+” mark indicates those peptides for which

production was confirmed but which were unable to be quantified due to the absence of a standard.

Table 7

Carboxy com- ponent	Amine com- ponent	Produced peptide	(mM)	Carboxy com- ponent	Amine com- ponent	Produced peptide	(mM)
Gly-OMe	L-CySH	Gly-L-CySH	45.6	L-Met-OMe	L-Ser	L-Met-L-Ser	12.8
	L-Arg	Gly-L-Arg	25.5		L-Met	L-Met-L-Met	25.0
	L-Phe	Gly-L-Phe	44.0		L-Phe	L-Met-L-Phe	34.0
	L-His	Gly-L-His	31.6	L-Ile-OMe	L-Ser	L-Ile-L-Ser	17.2
	L-Lys	Gly-L-Lys	9.8		L-Met	L-Ile-L-Met	10.0
	L-Ser	Gly-L-Ser	44.2		L-Phe	L-Ile-L-Phe	5.2
L-Thr-OMe	Gly	L-Thr-Gly	9.4	L-Arg-OMe	L-Ser	L-Arg-L-Ser	3.6
	L-Ala	L-Thr-L-Ala	9.4		L-Met	L-Arg-L-Met	0.7
	L-Val	L-Thr-L-Val	0.7		L-Phe	L-Arg-L-Phe	1.9
	L-Leu	L-Thr-L-Leu	28.4	L-Leu-OMe	L-Met	L-Leu-L-Met	12.2
	L-Met	L-Thr-L-Met	38.6	L-Trp-OMe	L-Met	L-Trp-L-Met	4.1
	L-Ser	L-Thr-L-Ser	58.2	L-Lys-OMe	L-Met	L-Lys-L-Met	6.8
L-Ser-OMe	L-Ser	L-Ser-L-Ser	38.0	L-His-OMe	L-Met	L-His-L-Met	6.5
	L-Met	L-Ser-L-Met	12.5	L-Asn-OMe	L-Glu	LAsn-L-Glu	10.2
	L-Phe	L-Ser-L-Phe	20.3				
L-Val-OMe	L-Ser	L-Val-L-Ser	30.8				
	L-Met	L-Val-L-Met	10.3				
	L-Phe	L-Val-L-Phe	6.1				

Hydrochloride salts were used for all of the carboxy components.

Example 14 Enzyme Substrate Specificity (5)

- 5 Peptide production was examined in the case of using the L or D forms of various amino acid methyl esters for the carboxy component, and the L or D forms of various amino acids for the amine component.

2 μ l of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the various amino acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 8 at 100 mM, the various amino acids shown in Table 8 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides produced in this reaction are shown in Table 8. (The “tr” indicates a trace amount.)

Table 8

Carboxy component	Amine component	Produced peptide	(mM)
D-Ala-OMe	L-Gln	D-Ala-L-Gln	69.3
D-Ala-OMe	L-Ser	D-Ala-L-Ser	20.3
D-Thr-OMe		D-Thr-L-Ser	1.0
D-Ser-OMe		D-Ser-L-Ser	3.3
D-Val-OMe		D-Val-L-Ser	0.6
D-Met-OMe		D-Met-L-Ser	5.1
L-Ala-OMe	D-Gln	L-Ala-D-Gln	0.3
L-Ala-OMe	D-Ser	L-Ala-D-Ser	5.4
L-Thr-OMe		L-Thr-D-Ser	6.9
L-Ser-OMe		L-Ser-D-Ser	16.2
L-Val-OMe		L-Val-D-Ser	1.4
L-Met-OMe		L-Met-D-Ser	1.9
D-Ala-OMe	D-Gln	D-Ala-D-Gln	tr
D-Ala-OMe	D-Ser	D-Ala-D-Ser	0.2
D-Thr-OMe		D-Thr-D-Ser	1.1
D-Ser-OMe		D-Ser-D-Ser	2.5
D-Val-OMe		D-Val-D-Ser	0.5
D-Met-OMe		D-Met-D-Ser	2.7

(Hydrochloride salts were used for all of the carboxy components)

Example 15 Enzyme Substrate Specificity (6)

- 5 Peptide production was examined using various L-amino acid amides for the carboxy component, and various L-amino acids for the amine component. A 2 μ l aliquot of the same enzyme fraction as that used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the L-amino acid amide salts (AA-NH₂HCl) shown in Table 9 at 100 mM, the

L-amino acids shown in Table 9 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 9.

Table 9

Carboxy component	Amine component	Produced peptide	(mM)
L-Phe-NH ₂	L-Gln	L-Phe-L-Gln	0.2
L-Phe-NH ₂	L-Ser	L-Phe-L-Ser	0.6
L-Ala-NH ₂	L-Gln	L-Ala-L-Gln	7.6
L-Ala-NH ₂	L-Met	L-Ala-L-Met	3.4
L-Ala-NH ₂	L-His	L-Ala-L-His	3.9
L-Thr-NH ₂	L-Gln	L-Thr-L-Gln	0.3

5

Example 16 Enzyme Substrate Specificity (7)

Peptide production was examined in the case of using various L-alanine methyl esters for the carboxy component and C-protected L-amino acids for the amine component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the L-alanine methyl ester (Ala-OMe·HCl) shown in Table 10 at 100 mM, the L-amino acid amide hydrochlorides shown in Table 10 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 10.

Table 10

Carboxy component	Amine component	Produced peptide	(mM)
L-Ala-OMe	Gly-NH ₂	L-Ala-Gly-NH ₂	7.4
	L-Ala-NH ₂	L-Ala-L-Ala-NH ₂	8.3
	L-Phe-NH ₂	L-Ala-L-Phe-NH ₂	12.2

Example 17 Enzyme Substrate Specificity (8)

Peptide production was examined in the case of using various amino acid methyl esters for the carboxy component and methylamine for the amine component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the amino acid methyl ester hydrochloride (AA-OMe·HCl) shown in Table 11 at 100 mM, the methylamine shown in Table 11 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 11.

Table 11

Carboxy component	Amine component	Produced peptide	(mM)
Gly-OMe	Methylamine	Gly-methylamine	1.1
L-Thr-OMe		L-Thr-methylamine	0.2
L-Ala-OMe		L-Ala-methylamine	0.3

Example 18 Enzyme Substrate Specificity (9)

Peptide production was examined in the case of using β -amino

acid ester for the carboxy component or β -amino acid for the amine component. A 2 μ l aliquot of the same enzyme fraction as used in Example 5 was added to 100 μ l of 100 mM borate buffer (pH 9.0) containing the carboxy components shown in Table 12 at 100 mM, the amine components shown in Table 12 at 150 mM and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 12. Note that the “tr” indicates a trace amount.

Table 12

Carboxy component	Amine component	Produced peptide	(mM)
Gly-OMe	β -Ala	Gly- β -Ala	2.2
Gly-OMe	β -Phe	Gly- β -Phe	0.4
L-Ala-OMe	β -Ala	Ala- β -Ala	7.7
L-Ala-OMe	β -Phe	Ala- β -Phe	1.4
L-Thr-OMe	β -Ala	Thr- β -Ala	3.2
L-Thr-OMe	β -Phe	Thr- β -Phe	1.4
β -Ala-OMe	L- α -Ala	β -Ala-L- α -Ala	tr
β -Ala-OMe	β -Ala	β -Ala- β -Ala	0.2
B-Ala-OMe	L-Gln	β -Ala-L-Gln	0.6
B-Ala-OMe	L-Ser	β -Ala-L-Ser	3.2

10 Hydrochloride salts were used for all of the carboxy components.

Example 19 Enzyme Substrate Specificity (10)

Oligopeptide production was examined in the case of using

As has been indicated in the aforementioned Examples 9 to 19, the present enzyme obtained from *Empedobacter brevis* strain FERM P BP-8113 (Depositary institution: National Institute for Advanced
5 Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) was determined to have an extremely broad substrate specificity.

Table 13

Carboxy component	Amine component	Produced peptide	(mM)
L-Ala-OMe	L-Ala	L-Ala-L-Ala	28.7
	L-Ala-L-Ala	L-Ala-L-Ala-L-Ala	57.5
	L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 1	44.5
	L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 2	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 3	34.8
	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 4	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 5	1.4*
	L-Ala-L-Gln	L-Ala-L-Ala-L-Gln	15.2
	Gly-L-Ala	L-Ala-Gly-L-Ala	25.9
	Gly-Gly	L-Ala-Gly-Gly	41.7
	L-His-L-Ala	L-Ala-L-His-L-Ala	55.9
	L-Leu-L-Ala	L-Ala-L-Leu-L-Ala	48.3
	L-Phe-L-Ala	L-Ala-L-Phe-L-Ala	49.7
	L-Phe-Gly	L-Ala-L-Phe-Gly	43.7
Gly-OMe	L-Ala-L-Tyr	Gly-L-Ala-L-Tyr	1.7
	Gly-L-Gln	Gly-Gly-L-Gln	7.2
	Gly-L-Tyr-L-Ala	Gly-Gly-L-Tyr-L-Ala SEQ ID NO: 6	44.2
L-Thr-OMe	Gly-Gly	L-Thr-Gly-Gly	83.0

*: Since the solubility of L-Ala-L-Ala-L-Ala-L-Ala-L-Ala was low, the carboxy component was used at a concentration of 10 mM and the amine component at 15 mM in this reaction system. The other conditions were the same as explained in the example. Hydrochloride salts were used for all of the carboxy components.

Example 20 Comparison of Ability to Catalyze Peptide Production with Known Enzymes

The peptide formation ability of the present enzyme was compared with that of known enzymes. Carboxypeptidase Y described in EP 278787A1 and the thiol endopeptidases (ficin, papain, bromelain and chymopapain) described in EP 359399B1 were used for the known enzymes, and they were used in the form of purified enzymes manufactured by Sigma. The enzyme uniformly purified in Example 3 was used for the source of the enzyme of the present invention. These enzymes were added to a reaction system in the amounts shown in Table 14 as protein. The reaction was carried out by adding enzyme to 100 μ l of borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride and 200 mM L-glutamine and allowing to react at 25°C. Furthermore, enzyme dissolved in 10 mM acetate buffer (pH 5.0) containing 1 mM EDTA was used for the carboxypeptidase, while enzyme dissolved in 10 mM acetate buffer (pH 5.0) containing 2 mM EDTA, 0.1 M KCl and 5 mM dithiothreitol was used for the thiol endopeptidase. The ratios of the formation rates of L-alanyl-L-glutamine by these enzymes are shown in Table 14.

As a result, the production of an extremely trace amount of L-alanyl-L-glutamine was observed even in the absence of enzyme, while a slight increase in the formation rate was observed in the lot where carboxypeptidase or thiol endopeptidase was added as compared with the lot where no enzyme was added. In contrast, an overwhelmingly faster rate of production of L-alanyl-L-glutamine was observed in the lot where the present enzyme was added, and that rate of production was about 5,000 to 100,000 times faster than

carboxypeptidase Y and thiol endopeptidase. As has been described above, the present enzyme was verified to have an extremely fast peptide formation rate unlike any enzyme in the prior art. Furthermore, in contrast to the enzyme of the present invention being a dimer having a molecular weight of about 75,000, since the molecular weight of carboxypeptidase Y has been reported to be about 61,000, while that of thiol endopeptidase has been reported to be about 23,000 to 36,000, the L-alanyl-L-glutamine formation rate per molecular weight is even greater for the enzyme of the present invention than that per unit weight indicated in the examples.

Table 14

Enzyme	Amount of enzyme added (protein mg/ml)	L-Ala-L-Gln production rate (mM/min)	Ratio of L-Ala-L-Gln production rate per enzyme unit weight
No enzyme	0	0.0006	
Carboxypeptidase Y	0.61	0.0257	0.0191
Ficin	2.60	0.0096	0.0017
Papain	2.30	0.0106	0.0021
Bromelain	2.80	0.0062	0.0010
Chymopapain	3.60	0.0100	0.0013
Enzyme of present invention	0.02	4.4000	100.0

Example 21 Production of L-Alanyl-L-Glutamine Using Microbial Cells of *Sphingobacterium* sp.

A 50 ml medium (pH7.0) containing 5 g of glucose, 5 g of

ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 g of peptone in 1 L was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes for culturing

5 *Sphingobacterium sp.* strain FERM BP-8124 (Depository institution: National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002). This medium was

10 then inoculated with one loopful of *Sphingobacterium sp.* strain FERM BP-8124 (Depository institution: National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date:

15 July 22, 2002) cultured at 30°C for 24 hours in a slant agar medium (agar: 20 g/L, pH 7.0) containing 5 g of glucose, 10 g of yeast extract, 10 g of peptone and 5 g of NaCl in 1 L, followed by shake culturing at 30°C for 20 hours and 120 strokes/minute. A 1 ml aliquot of this culture broth was then added to the aforementioned medium (50 ml/500

20 mL Sakaguchi flask) and cultured at 30°C for 18 hours. Following completion of the culturing, the microbial cells were separated from the culture broths by centrifugation and suspended in 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells. A 0.1 mL aliquot of 100 mM borate buffer (pH 9.0) containing 10 mM

25 EDTA, 200 mM L-alanyl methyl ester hydrochloride and 400 mM

L-glutamine was then added to 0.1 mL of this microbial cell suspension, and after bringing to a final volume of 0.2 mL, was allowed to react at
5 25°C for 120 minutes. The amount of L-alanyl-L-glutamine formed at this time was 62 mM.

Example 22 Purification of Enzyme from *Sphingobacterium* sp.

The following procedure after centrifugal separation was carried
10 out either on ice or at 4°C. *Sphingobacterium* sp. strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi,
15 Ibaraki-ken, Japan, International deposit date: July 22, 2002) was cultured in the same manner as Example 21, and the microbial cells were collected by centrifugal separation (10,000 rpm, 15 minutes). After washing 2 g of microbial cells with 20 mM Tris-HCl buffer (pH 7.6), they were suspended in 8 ml of the same buffer and subjected to
20 ultrasonic crushing treatment for 45 minutes at 195 W. This ultrasonic crushing liquid was then centrifuged (10,000 rpm, 30 minutes) to remove the crushed cell fragments and obtain an ultrasonic crushing liquid supernatant. This ultrasonic crushing liquid supernatant was dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.6) followed by
25 removal of the insoluble fraction by ultracentrifugation (50,000 rpm, 30

minutes) to obtain a soluble fraction in the form of the supernatant liquid. The resulting soluble fraction was applied to a Q-Sepharose HP column (manufactured by Amersham) pre-equilibrated with Tris-HCl buffer (pH 7.6), and the active fraction was collected from the

5 non-adsorbed fraction. This active fraction was dialyzed overnight against 20 mM acetate buffer (pH 5.0) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the supernatant liquid. This dialyzed fraction was then applied to an SP-Sepharose HP column

10 (manufactured by Amersham) pre-equilibrated with 20 mM acetate buffer (pH 5.0) to obtain the active fraction in which enzyme was eluted at a linear concentration gradient of the same buffer containing 0 to 1 M NaCl.

15 **Example 23 Production of L-Alanyl-L-Glutamine Using Enzyme Fraction**

A 10 μ l aliquot of the SP-Sepharose HP fraction (about 27 U/ml) purified in Example 22 was added to 90 μ l of 111 mM borate buffer (pH 9.0) containing 111 mM L-alanine methyl ester hydrochloride, 222 mM L-glutamine and 11 mM EDTA, and allowed to react at 25°C for 120

20 minutes. As a result, 73 mM of L-alanyl-L-glutamine was formed in the enzyme-added lot. On the other hand, there was hardly any production of L-Ala-L-Glu observed in the enzyme-non-added lot, and the production amount was only about 0.07 mM after reacting for 120 minutes.

Example 24 Enzyme Substrate Specificity (11)

Substrate specificity was examined for enzyme derived from *Sphingobacterium* sp. strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002). 100 μ l of 100 mM borate buffer (pH 9.0) containing the various carboxy components at a final concentration of 100 mM and the various amine components at a final concentration of 150 mM shown in Tables 15-1 to 15-4, the SP-Sepharose HP fraction enzyme purified in Example 22 (addition of 0.33 units in the reaction liquid) and 10 mM EDTA were allowed to react at 25°C for 1.5 hours. The amounts of each of the peptides formed in this reaction are shown in Table 15. Note that the “+” mark indicates those peptides for which production was confirmed but which were unable to be quantified due to the absence of a standard, while “tr” indicates a trace amount.) Furthermore, Tween-80 was added to the reaction system to a final concentration of 0.1% in the case of using L-Tyr-OMe. In addition, hydrochlorides were used for all carboxy components.

Table 15-1

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	Gly	L-Ala-Gly	11.1
	L-Ala	L-Ala-L-Ala	13.1
	L-Val	L-Ala-L-Val	10.9
	L-Leu	L-Ala-L-Leu	33.0
	L-Ile	L-Ala-L-Ile	24.7
	L-Met	L-Ala-L-Met	86.9
	L-Pro	L-Ala-L-Pro	1.5
	L-Phe	L-Ala-L-Phe	69.5
	L-Trp	L-Ala-L-Trp	46.0
	L-Thr	L-Ala-L-Thr	47.3
	L-Asn	L-Ala-L-Asn	52.3
	L-Tyr	L-Ala-L-Tyr	11.1
	L-CySH	L-Ala-L-CySH	+
	L-Lys	L-Ala-L-Lys	71.2
	L-Arg	L-Ala-L-Arg	72.2
	L-His	L-Ala-L-His	73.6
	L-Asp	L-Ala-L-Asp	2.3
	L-Glu	L-Ala-L-Glu	39.1
	L-Ser	L-Ala-L-Ser	43.8
	D-Ser	L-Ala-D-Ser	3.3
D-Ala-OMe	L-Ser	D-Ala-L-Ser	24.1
	D-Ser	D-Ala-D-Ser	5.5

Table 15-2

Carboxy component	Amine component	Produced peptide (mM)	
L-Thr-OMe	L-Gln	L-Thr-L-Gln	36.1
Gly-OMe		Gly-L-Gln	61.1
L-Ser-OMe		L-Ser-L-Gln	12.9
L-Val-OMe		L-Val-L-Gln	8.2
L-Met-OMe		L-Met-L-Gln	32.6
L-Ile-OMe		L-Ile-L-Gln	6.4
L-Arg-OMe		L-Arg-L-Gln	17.2
L-Tyr-OMe		L-Tyr-L-Gln	0.6
L-Pro-OMe		L-Pro-L-Gln	1.8
L-Phe-OMe		L-Phe-L-Gln	0.8
L-Gln-OMe		L-Gln-L-Gln	0.1
Asp- α -OMe		α -L-Asp-L-Gln	0.05

Table 15-3

Carboxy component	Amine component	Produced peptide (mM)	
L-Thr-OMe	Gly	L-Thr-Gly	0.4
	L-Ala	L-Thr-L-Ala	5.8
	L-Val	L-Thr-L-Val	1.3
	L-Leu	L-Thr-L-Leu	15.3
	L-Met	L-Thr-L-Met	28.9
Gly-OMe	L-Arg	Gly-L-Arg	17.9
	L-Phe	Gly-L-Phe	20.0
	L-His	Gly-L-His	36.2
	L-Lys	Gly-L-Lys	48.2
	L-Ser	Gly-L-Ser	53.8
L-Ser-OMe	L-Ser	L-Ser-L-Ser	9.9
	L-Met	L-Ser-L-Met	7.6
	L-Phe	L-Ser-L-Phe	4.3
L-Val-OMe	L-Ser	L-Val-L-Ser	31.9
	L-Met	L-Val-L-Met	6.8
	L-Phe	L-Val-L-Phe	1.0
L-Met-OMe	L-Ser	L-Met-L-Ser	25.3
	L-Met	L-Met-L-Met	28.4
	L-Phe	L-Met-L-Phe	8.9
L-Ile-OMe	L-Ser	L-Ile-L-Ser	17.3
	L-Met	L-Ile-L-Met	5.1
	L-Phe	L-Ile-L-Phe	1.5
L-Arg-OMe	L-Ser	L-Arg-L-Ser	2.2
	L-Met	L-Arg-L-Met	tr
	L-Phe	L-Arg-L-Phe	tr

Table 15-4

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	Gly amide	L-Ala-Gly amide	15.1
	L-Ala amide	L-Ala-L-Ala amide	9.2
	L-Phe amide	L-Ala-Phe amide	27.1
L-Ala-OMe	Methylamine	L-Ala-methylamine	0.6
L-Thr-OMe		L-Thr-methylamine	0.3
Gly-OMe		Gly-methylamine	1.0
L-Ala amide	L-Gln	L-Ala-L-Gln	0.3
	L-Met	L-Ala-L-Met	tr
	L-His	L-Ala-L-His	tr

(Hydroxychloride salts were used for all the amino acid amides.)

Example 25 Enzyme Substrate Specificity (12)

- 5 Substrate specificity with respect to oligopeptide production was examined for enzyme derived from *Sphingobacterium sp.* strain FERM BP-8124 (Depository institution: the independent administrative corporation, National Institute for Advanced Industrial Science and Technology, International Patent Organism Depository, Address of
- 10 depository institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002). A 100 μ l aliquot of 100 mM borate buffer (pH 9.0) containing the various carboxy components at a final concentration of 100 mM and the various amine components at a final concentration of 150 mM shown in Table 16, the

SP-Sepharose HP fraction enzyme purified in Example 22 (addition of 0.33 units in the reaction liquid) and 10 mM EDTA were allowed to react for 1.5 hours at 25°C. The amounts of each oligopeptide formed in this reaction are shown in Table 16. Furthermore, hydrochloride salts were
 5 used for all carboxy components.

Table 16

Carboxy component	Amine component	Produced peptide	(mM)
L-Ala-OMe	L-Ala	L-Ala-L-Ala	25.6
	L-Ala-L-Ala	L-Ala-L-Ala-L-Ala	41.1
	L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 7	30.1
	L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 8	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala SEQ ID NO: 9	22.8
	Gly-Gly	L-Ala-Gly-Gly	33.7
	Gly-Ala	L-Ala-Gly-L-Ala	35.1
	L-His-L-Ala	L-Ala-L-His-L-Ala	58.0
	L-Phe-Gly	L-Ala-L-Phe-Gly	34.0
	L-Leu-L-Ala	L-Ala-L-Leu-L-Ala	40.7
	L-Phe-L-Ala	L-Ala-L-Phe-L-Ala	24.8
L-Thr-OMe	Gly-Gly	L-Thr-Gly-Gly	8.4
Gly-OMe	L-Ala-L-Tyr	Gly-L-Ala-L-Tyr	0.6

Example 26 Enzyme Substrate Specificity (13)

Substrate specificity was additionally assessed using the same
 10 enzyme fraction as that used in Example 5.

Table 17

Carboxy component (mM)	Amine component (mM)	Produced peptide (mM)	Reaction time (hr)
H-Ala-OMe 50mM	H-p-F-Phe-OH 50mM	H-Ala-p-F-Phe-OH 21.9mM	3
H-Ala-OMe 40mM	H-Cl-F-Phe-OH 40mM	H-Ala-Cl-F-Phe-OH 20.8mM	3
H-Ala-OMe 40mM	H-p-NO ₂ -Phe-OH 40mM	H-Ala-p-NO ₂ -Phe-OH 27.5mM	3
H-Ala-OMe 100mM	H-t-Leu-OH 150mM	H-Ala-t-Leu-OH 0.4mM	3
H-Ala-OMe 20mM	H-2-Nal-OH 20mM	H-Ala-2-Nal-OH +	3
H-p-F-Phe-OMe 100mM	H-Gln-OH 150mM	H-p-F-Phe-H-Gln-OH tr	3
H-Cl-F-Phe-OMe 25mM	H-Gln-OH 50mM	H-Cl-F-Phe-H-Gln-OH tr	3
H-p-NO ₂ -Phe-OMe 40mM	H-Gln-OH 40mM	H-p-NO ₂ -Phe-H-Gln-OH 1.1mM	3
H-t-Leu-OMe 100mM	H-Gln-OH 150mM	H-t-Leu-H-Gln-OH tr	3
H-2-Nal-OMe 40mM	H-Gln-OH 40mM	H-2-Nal-H-Gln-OH tr	3
H-Aib-OMe 100mM	H-Gln-OH 150mM	H-Aib-H-Gln-OH 18.8mM	3
H-N-Me-Ala-OMe 100mM	H-Gln-OH 150mM	H-N-Me-Ala-H-Gln-OH 0.5mM	3
H-Aib-OMe 100mM	H-Phe-OH 150mM	H-Aib-Phe-OH 17.2mM	3
H-CHA-OMe 40mM	H-Phe-OH 40mM	H-CHA-Phe-OH +	3
H-N-Me-Ala-OMe 100mM	H-Phe-OH 150mM	H-N-Me-Ala-Phe-OH tr	3
H-Ala-OMe 100mM	H-Ser(tBu)-OH 150mM	H-Ala-Ser(tBu)-OH 48.8mM	2
H-Ser(tBu)-OMe 100mM	H-Gln-OH 150mM	H-Ser(tBu)-Gln-OH tr	2
H-Ala-OMe 100mM	H-Asp(OtBu)-OH 150mM	H-Ala-Asp(OtBu)-OH 62.6mM	2
H-Asp(OtBu)-OMe 100mM	H-Gln-OH 150mM	H-Asp(OtBu)-Gln-OH 0.9mM	2
H-Ala-OMe 100mM	H-Lys(Boc)-OH 150mM	H-Ala-Lys(Boc)-OH 51.0mM	2
H-Lys(Boc)-OMe 100mM	H-Gln-OH 150mM	H-Lys(Boc)-Gln-OH +	2

100 μ l of reaction solutions consisting of 100 mM borate buffer (pH 9.0) containing each of the carboxy components and amine components at the final concentrations shown in Table 17, enzyme (addition of 0.1 unit in reaction solution) and 10 mM EDTA were allowed to react at 25°C for the reaction times shown in Table 17. The amounts of each of the peptides produced in the reactions are shown in Table 17. (A "+" mark indicates those for which production was confirmed but which were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount).

5 Abbreviations:

- H-Ala-OMe: L-alanine methyl ester hydrochloride
- H-p-F-Phe-OMe: p-fluoro-L-phenylalanine methyl ester hydrochloride
- H-Cl-F-Phe-OMe: p-chloro-L-phenylalanine methyl ester hydrochloride
- H-p-NO₂-Phe-OMe: p-nitro-L-phenylalanine methyl ester hydrochloride
- 10 H-t-Leu-OMe: tert-L-leucine methyl ester hydrochloride
- H-2-Nal-OMe: 3-(2-naphthyl)-L-alanine methyl ester hydrochloride
- H-Aib-OMe: α -aminoisobutyric acid methyl ester hydrochloride
- H-N-Me-Ala-OMe: N-methyl-L-alanine methyl ester hydrochloride
- H-CHA-OMe: β -cyclohexyl-L-alanine methyl ester hydrochloride
- 15 H-Ser(tBu)-OMe: O-tert-butyl-L-serine methyl ester hydrochloride
- H-Asp(OtBu)-OMe: L-aspartic acid β -tert-butyl ester α -methyl ester hydrochloride
- H-Lys(Boc)-OMe: N- ϵ -tert-butoxycarbonyl-L-lysine methyl ester hydrochloride
- 20 H-p-F-Phe-OH: p-fluoro-L-phenylalanine
- H-Cl-F-Phe-OH: p-chloro-L-phenylalanine
- H-p-NO₂-Phe-OH: p-nitro-L-phenylalanine
- H-t-Leu-OH: tert-L-leucine
- H-2-Nal-OH: 3-(2-naphthyl)-L-alanine
- 25 H-Gln-OH: L-glutamine

H-Phe-OH: L-phenylalanine

H-Ser(tBu)-OH: O-tert-butyl-L-serine

H-Asp(OtBu)-OH: L-aspartic acid β -tert-butyl ester

H-Lys(Boc)-OH: N- ϵ -tert-butoxycarbonyl-L-lysine

5

Example 27 Enzyme Substrate Specificity (14)

Substrate specificity with respect to oligopeptide production was assessed using the same enzyme fraction as Example 26. 100 μ l of reaction solutions consisting of 100 mM borate buffer (pH 9.0) containing each of the carboxy components and amine components at the final concentrations shown in Table 18, enzyme (the numbers of units added to the reaction solution are described in Table 18) and 10 mM EDTA were allowed to react at 25°C for 3 hours. The amounts of each of the oligopeptides formed in the reactions are shown in Table 18. Note that the “+” mark indicates those for which production was confirmed but which were unable to be quantified due to the absence of a standard, while “tr” indicates a trace amount). It should be noted that hydrochlorides were used for all of the carboxy components.

10

15

Table 18

Carboxy component	Amine component	Amount of enzyme (units)	Produced peptide (mM)	
Gly-OMe	L-Phe-L-Met	1.0	Gly-Phe-Met	13.3
L-Ala-OMe	L-Phe-L-Met	0.2	L-Ala-L-Phe-L-Met	+
L-Tyr-OMe	Gly-Gly-L-Phe-L-Met SEQ ID NO: 10	1.0	L-Tyr-Gly-Gly-L-Phe-L-Met SEQ ID NO: 11	2.7
L-Ala-OMe	Gly-Gly-L-Phe-L-Met SEQ ID NO: 12	0.2	L-Ala-Gly-Gly-L-Phe-L-Met SEQ ID NO: 13	+
Gly-OMe	Gly-L-Phe	0.1	Gly-L-Phe	17.3
L-Ala-OMe	Gly-L-Phe	0.1	L-Ala-Gly-L-Phe	+
D-Ala-OMe	Gly-L-Phe	0.1	D-Ala-Gly-L-Phe	Tr

INDUSTRIAL APPLICABILITY

The present invention provides a novel enzyme with which a peptide can be produced easily, inexpensively and at high yield by mitigating use of complex synthesis methods such as introduction and elimination of protecting groups. The use of the enzyme of the present invention enables efficient industrial production of peptides.

CLAIMS

1. An enzyme isolated from a microbe belonging to a species selected from the group consisting of *Empedobacter brevis* and *Sphingobacterium sp.*, and having the ability to form a peptide from a carboxy component and an amine component;

wherein the enzyme has a molecular weight as determined by SDS-gel electrophoresis of about 75 kilodaltons, and a molecular weight as determined by gel filtration chromatography of about 150 kilodaltons;

wherein the enzyme has the ability to form a peptide within a pH range of 6.5 to 10.5;

wherein the enzyme has the ability to form a peptide within a temperature range of 5 to 60°C;

wherein the enzyme has the ability to form a peptide from a carboxy component and an amine component and the ability to form L-alanyl-L-glutamine at a formation rate of 0.03 mM/min or more in a dipeptide-forming reaction under conditions (i) to (iv):

(i) the carboxy component is L-alanine methyl ester hydrochloride in an amount of 100 mM;

(ii) the amine component is L-glutamine in an amount of 200 mM;

(iii) the pH is 9.0; and

(iv) the amount of homogeneously purified enzyme added is less than 0.61 mg/ml as protein amount.

2. The enzyme according to claim 1, wherein the carboxy

component as a substrate is an amino acid ester or an amino acid amide.

3. The enzyme according to claim 1, wherein the amine component as a substrate is an amino acid, a C-protected amino acid or an amine.

4. The enzyme according to claim 1, wherein the enzyme is not inhibited by the serine enzyme inhibitor, phenylmethanesulfonyl fluoride, but is inhibited by p-nitrophenyl-p'-guanidinobenzoate.

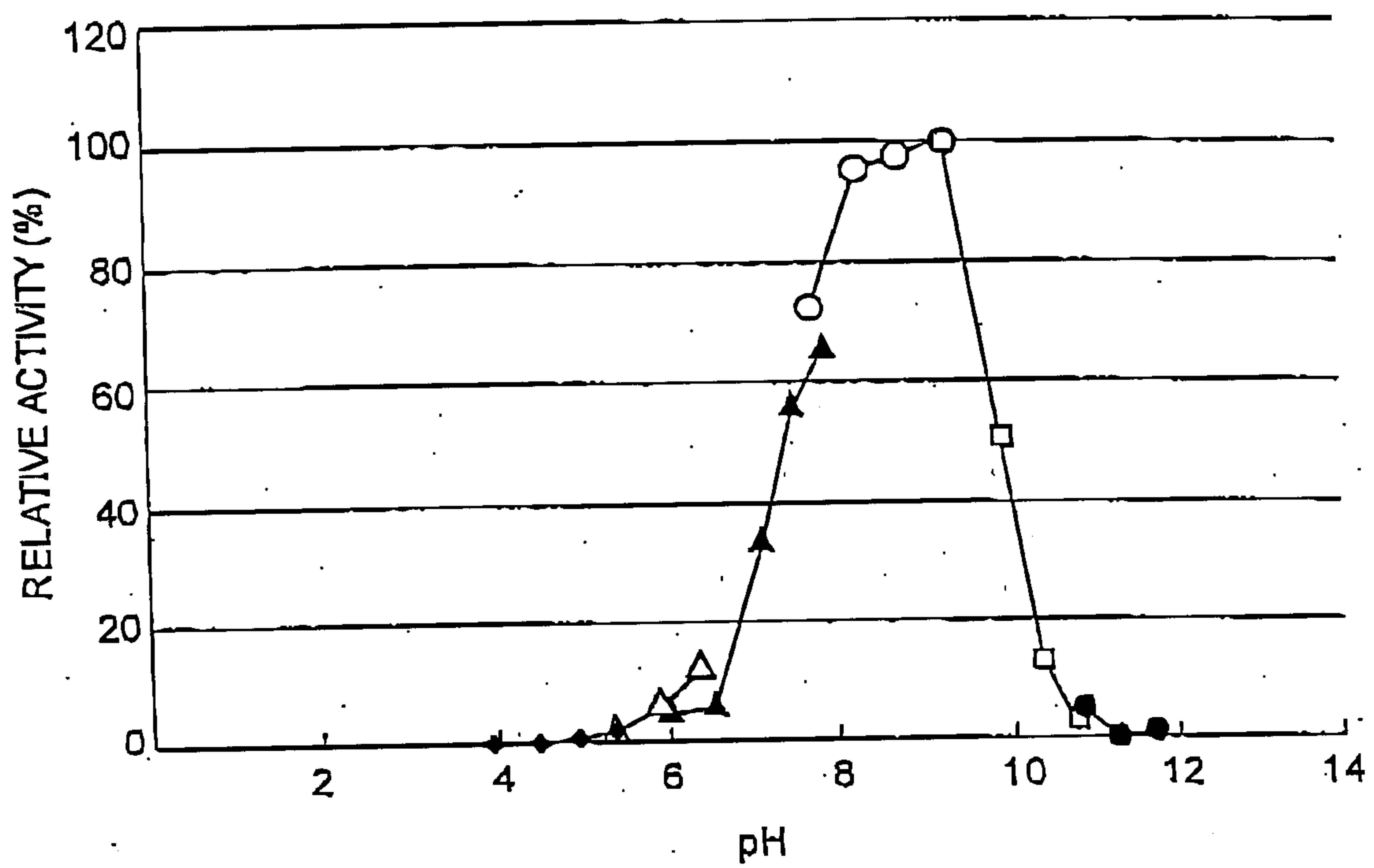
5. A microbe from biological deposit *Empedobacter brevis* strain FERM BP-8113 or *Sphingobacterium sp.* strain FERM BP-8124.

6. A method for producing a dipeptide comprising:

- mixing a carboxy component and an amine component in the presence of an enzyme according to claim 1 and/or a substance containing the enzyme,
- reacting the carboxy component with the amine component to form the dipeptide, and
- recovering the dipeptide.

1/3

FIG.1



- ◆ : ACETATE BUFFER (pH3.9~5.4)
- △ : MES BUFFER (pH5.4~6.4)
- ▲ : PHOSPHATE BUFFER (pH6.0~7.9)
- : BORATE BUFFER (pH7.8~9.3)
- : CAPS BUFFER (pH9.3~10.7)
- : K₂HPO₄-NaOH BUFFER (pH10.8~11.6)

2/3

FIG.2

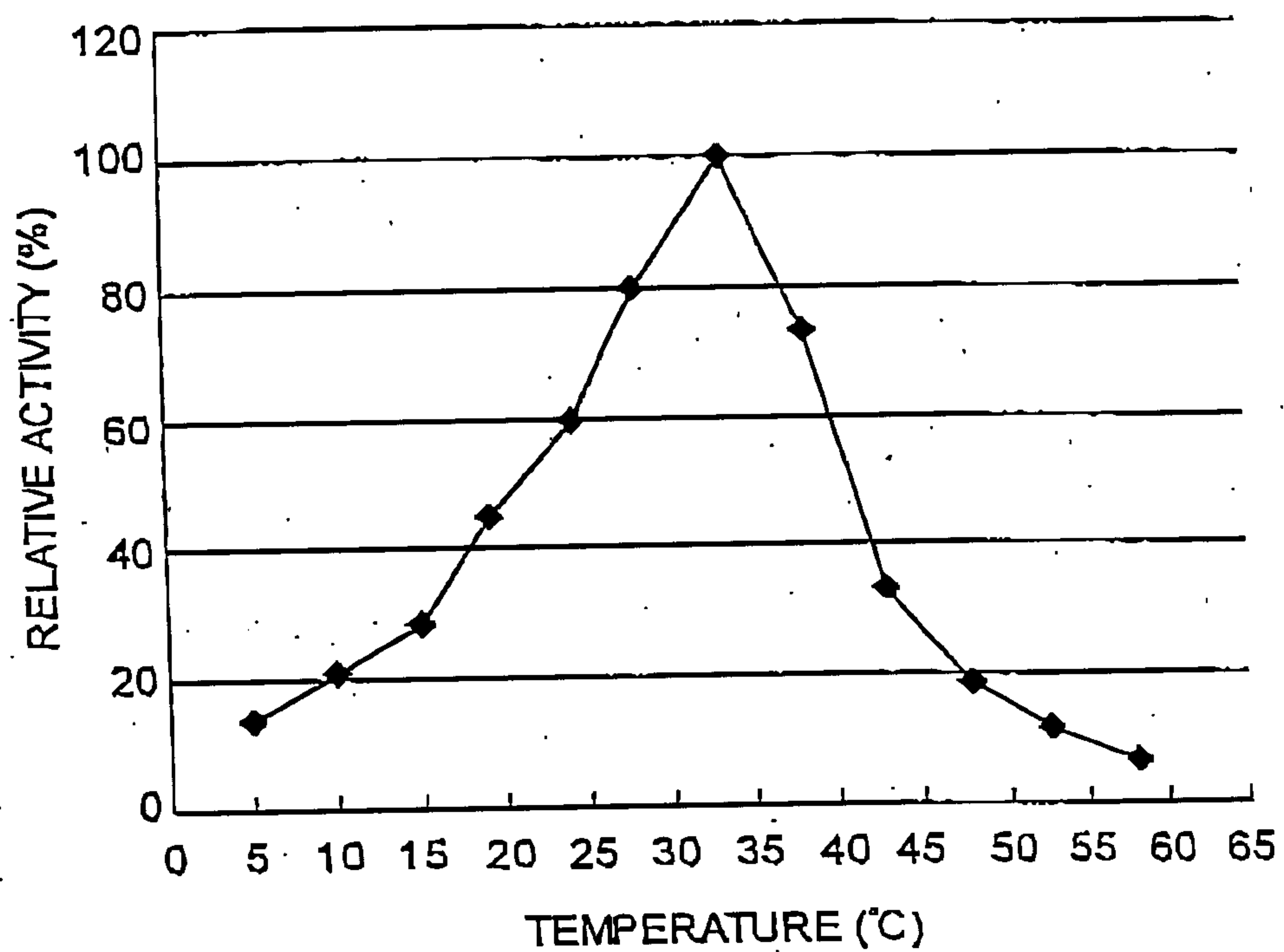
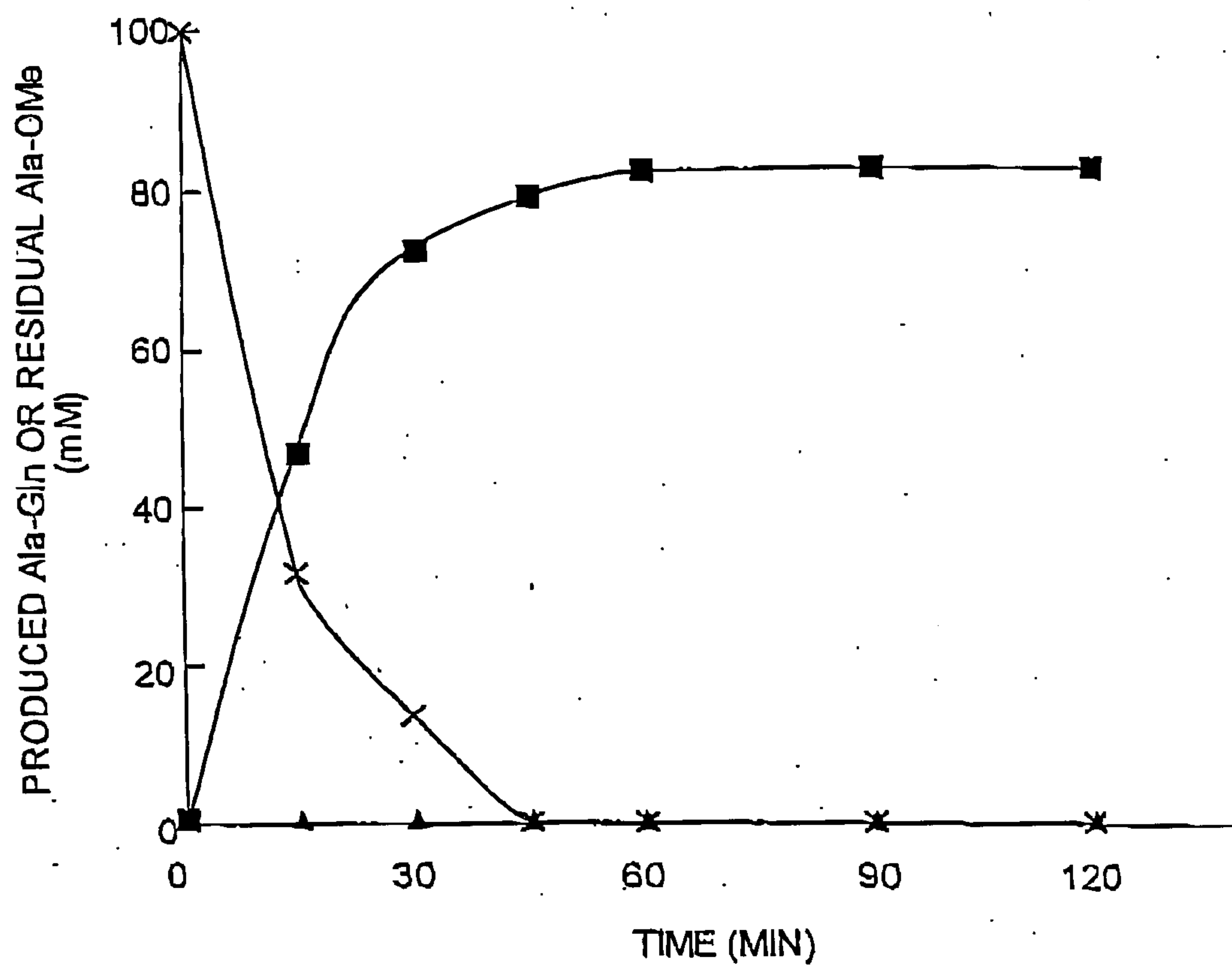
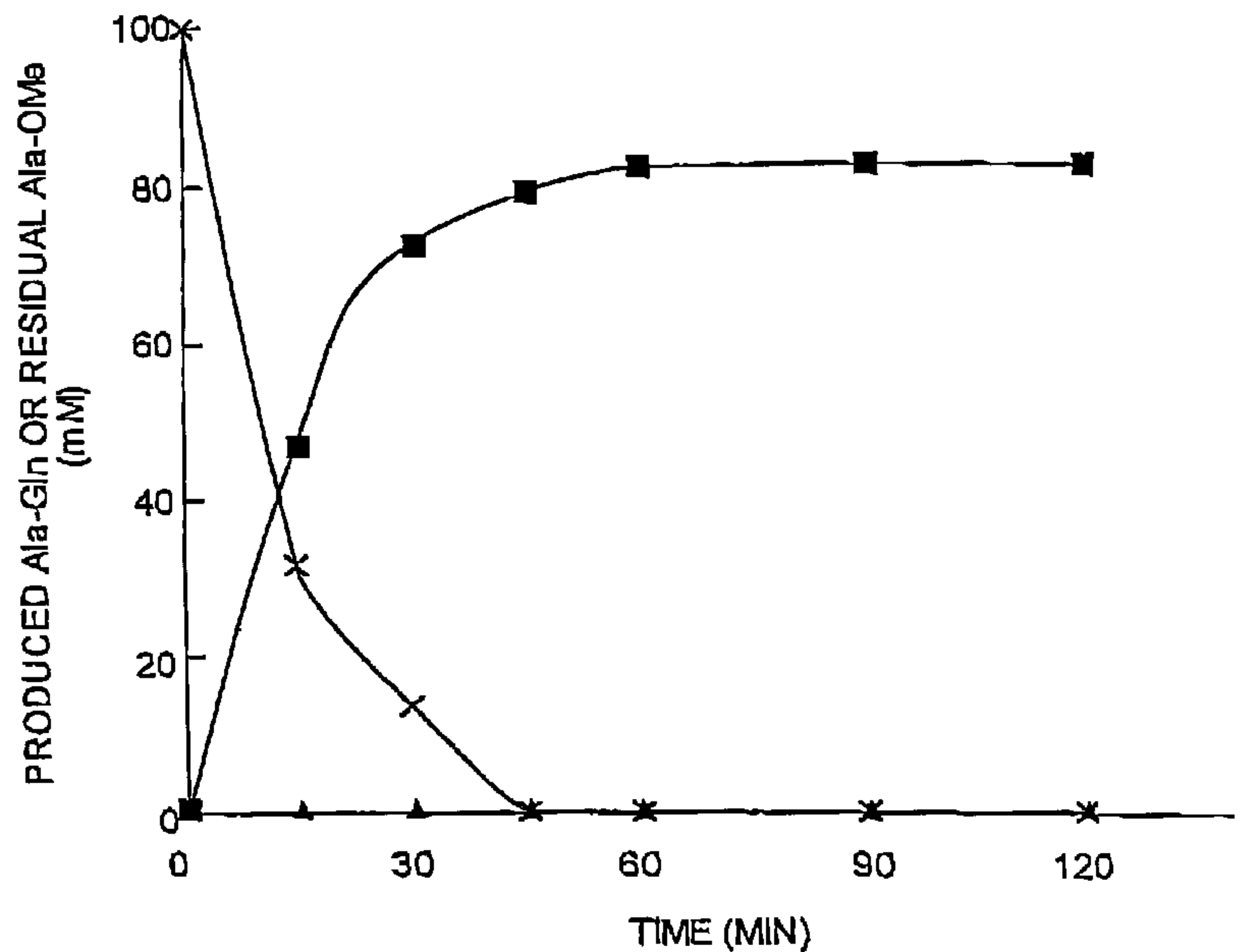


FIG.3



- : L-Ala-L-Gln PRODUCED IN ENZYME - ADDED LOT
X : RESIDUAL L-Ala-OMe IN ENZYME - ADDED LOT
▲ : L-Ala-L-Gln PRODUCED IN ENZYME - NON - ADDED LOT



■ : L-Ala-L-Gln PRODUCED IN ENZYME - ADDED LOT

× : RESIDUAL L-Ala-OMe IN ENZYME - ADDED LOT

▲ : L-Ala-L-Gln PRODUCED IN ENZYME - NON - ADDED LOT