(57) Abrégé/Abstract:
It is intended to provide an actinomyces-origin neutral metalloprotease, which selectively cleaves the pro-structure moiety of a microorganism-origin protransglutaminase, and a gene encoding the same. An activated microorganism-origin transglutaminase from which the pro-structural moiety has been excised can be produced by culturing a microorganism having the gene encoding the above-described actinomyces-origin neutral metalloprotease transferred thereinto, allowing the microorganism to produce the actinomyces-origin neutral metalloprotease and then treating the microorganism-origin protransglutaminase therewith.
Abstract of the Disclosure

The present invention provides a neutral metalloprotease from actinomycetes which selectively cleaves a pro-structure part of a microbial protransglutaminase and a gene encoding. An active microbial transglutaminase having the pro-structure part cleaved can be obtained by culturing a microorganism into which a gene encoding the neutral metalloprotease from actinomycetes according to the present invention has been introduced, where by producing the neutral metalloprotease from actinomycetes, and reacting it on a microbial protransglutaminase.
METHOD OF PRODUCING MICROBIAL TRANSGlutaminase

Field of the invention

The present invention relates to a new protease which efficiently cleaves the pro-
structure part of pro-transglutaminase to convert to an active form transglutaminase and to
a nucleic acid encoding it, said pro-transglutaminase being produced by actinomycetes.

The present invention also relates to a method of producing microbial
transglutaminase in its active form using said protease.
Additionally, the present invention relates to a method of producing the neutral
metalloprotease.

Background of the invention

Transglutaminase is an enzyme which catalyzes acyltransfer reaction of \( \gamma \)-
carboxylamide groups in the peptide chain of the protein. When the enzyme is reacted with
a protein, the formation of the cross-linkage \( \varepsilon -(\gamma-\text{Glu})-\text{Lys} \) and the replacement of Gin
with Glu by deamidation can be occurred. Transglutaminases have been used to
manufacture gelled food products such as jelly, yogurt, cheese or gelled cosmetics and
others, to improve the quality of meat, etc. (Japanese publication of examined
application (JP-Kokoku) No. 1-50382). Moreover, transglutaminase is an enzyme having
industrially high usefulness in that it has been used to manufacture materials for
thermostable microcapsules, carrier for immobilized enzyme etc.

Transglutaminases from animals, which are calcium-dependent in expressing the
activities, and transglutaminases from microorganisms (microbial transglutaminase(s),
which is/are also referred to as "MTG(s)" hereinafter), which are calcium-independent in
expressing the activities, have been previously known. For MTG, a transglutaminase from a
bacterium belonging to genus Streptoverticillium has been discovered. Such
Streptoverticillium bacteria include, for example, Streptoverticillium griseocarneum IFO
12776, Streptoverticillium cinnamoneum sub sp. cinnamoneum IFO 12852,
Streptoverticillium mobaraense (hereinafter it may abbreviated as S. mobaraense) IFO
Because these transglutaminases, however, have been produced through the purification from the cultures such as those of microorganisms described above, there have been problems in terms of the amount and the efficiency and the like. Then, as a method for secreting heterologous proteins efficiently, the method was established wherein a coryneform bacterium is selected as a host, a fused protein having transglutaminase connected downstream to the signal peptide domain of a coryneform bacterium, and the transglutaminase was efficiently secreted to obtain high yield of transglutaminase (WO 01/23591). In this study, a method is described wherein MTG is secreted in an inactive form as pro-transglutaminase (referred to as "pro MTG" hereinafter) in which a pro-structure part is connected to MTG, and then the pro-structure part of the pro MTG is cleaved by a protease to convert it into a transglutaminase having activity, as well as a method wherein an active transglutaminase is directly produced in the culture medium by co-expressing SAM-P45, which is a serine protease derived from actinomycetes, at a required and sufficient amount in a coryneform bacterium producing the pro MTG.

Although a method in which an active transglutaminase is directly produced by co-expressing pro MTG and a protease which allows to cleave the pro-structure part of the pro MTG in a coryneform bacterium is assumed to be an extremely efficient method of producing transglutaminase, the substrate specificity of SAM-P45 is not so strict and it may digest and degrade not only the pro-structure part of the pro MTG but also the transglutaminase itself in some degree, the handling of SAM-P45 therefore may not be easy. In the cases where SAM-P45 is used, therefore, the production method of transglutaminase should be strictly controlled such that the decomposition of the produced transglutaminase in the culture medium will not occur.

There has been still remained, therefore, a demand for a protease which can selectively cleave only the pro-structure part of pro MTG, and cause an over-decomposition of the transglutaminase itself as little as possible for performing the production of an active form transglutaminase advantageously.

As an enzyme which cleaves the pro-structure part of pro MTG, besides SAM-P45, a dispase derived from *Bacillus polymyxa* is known (Eur. J. Biochem., vol. 257, p. 570-576...
(1998)). A large amount of enzymes, however, are required for the cleavage of the pro-
structure part, and there is a risk of the over-decomposition of the transglutaminase itself. In
addition, a dispase is the reagent for cell culture, so it is expensive as an enzyme for
industrial use.

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Summary of the Invention

There has been still remained a need for a protease which can selectively cleave
only the pro-structure part of pro MTG, and cause an over-decomposition of the
transglutaminase itself as little as possible for performing the production of an active form
transglutaminase advantageously, as mentioned above. Additionally, if proteases could be
used which can selectively cleave only the pro-structure part of pro MTG, and cause an
over-decomposition of the transglutaminase itself as little as possible, it was believed to be
advantageous for the production of an active transglutaminase. Furthermore, if the useful
proteases for the production of transglutaminase which can selectively cleave the pro-
structure part of pro MTG were those which could be secreted extracellularly, it was
believed to be more preferable because active transglutaminases could be directly
produced in the culture medium by co-expressing them together with the pro MTG.

Therefore, an object of the present invention is to provide a protease which is
useful for the production of transglutaminase and which selectively cleaves the pro-
structure part of pro MTG.

In particular, an object of the invention is to provide protease which selectively
cleaves the pro-structure part of pro MTG, wherein said protease can be produced by using
a coryneform bacterium as a host, and can be easily secreted extracellularly.

An object of the invention is also to provide a nucleic acid molecule encoding said
protease.

Another object of the invention is to provide a method of producing MTG efficiently
using said protease.

Furthermore, an object of the invention is to provide a method of producing said
protease.

The inventors of the present invention searched a protease which would selectively
cleave the pro-structure part of pro MTG, but would cause the decomposition of the transglutaminase itself as little as possible, and then they could isolate and purify a neutral metalloprotease having such a property. The inventors also obtained a DNA encoding said protease, introduced it into a coryneform bacterium, and then they were successful in its secretory expression using a coryneform bacterium as a host. In addition, the present enzyme was actually reacted on the pro MTG to cleave the pro-structure part, and then the active transglutaminase was recovered. The inventors established the present invention by identifying neutral metalloproteases derived from microorganisms of other sources having an equivalent function, which have been proved similarly useful for the production of an active MTG.

Namely, the present invention is a neutral metalloprotease from actinomycetes having high selectivity in cleaving the pro-structure part of pro MTG, and a nucleic acid molecule encoding it.

The present invention is also a method of producing an active MTG, comprising cleaving a pro-structure part of a pro MTG by a neutral metalloprotease.

The present invention is also a method of producing said metalloprotease, comprising introducing a nucleic acid molecule encoding said neutral metalloprotease into a coryneform bacterium, culturing the coryneform bacterium into which said nucleic acid molecule has been introduced, thereby allowing the expression of said neutral metalloprotease, and recovering said extracellularly secreted metalloprotease.

More specifically, the present invention is a neutral metalloprotease SVP35 from actinomycetes having the following properties:
1) Molecular weight: about 35,000 (as measured by SDS-PAGE)
2) Optimum pH: 6.0-8.0, more specifically 6.5-7.5, in particular around 7.0
3) pH Stability: pH of 4-10
4) Optimum temperature: about 45°C
5) Temperature stability: it is stable below about 50°C
6) It is strongly inhibited by ethylene diamine tetraacetic acid, 1,10- phenanthroline and phosphoramidon which are metalloprotease inhibitors, and by Streptomyces subtilisin inhibitor (SSI) from actinomycetes.
The present invention is also a neutral metalloprotease SVP70 having the following properties:

1) Molecular weight: about 71,000 (as measured by SDS-PAGE)
2) Optimum pH: the range of 6.0-8.0, more specifically 6.5-7.5, in particular around 7.0
3) pH Stability: pH of 5-10
4) Optimum temperature: the range of about 50°C-55°C, in particular around 55°C
5) It undergoes a strong inhibitory action by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, dithiothreitol which is a SH-reductant, and by Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes.

The present invention is also a nucleic acid molecule encoding said SVP35 or SVP70.

The present invention is also a method of producing an active MTG, comprising cleaving the pro-structure part of pro MTG by said SVP35 or SVP70.

Furthermore, the present invention is a method of producing SVP35 or SVP70, comprising introducing a nucleic acid molecule encoding said SVP35 or SVP70 into a coryneform bacterium, culturing the coryneform bacterium into which said nucleic acid molecule has been introduced, and recovering the extracellularly secreted SVP35 or SVP70.

**Brief Description of the Drawings**

Figure 1 is a graph exhibiting the pH dependence of SVP35 and SVP70 activity.

Figure 2 is a graph exhibiting the pH stability of SVP35 and SVP70.

Figure 3 is a graph exhibiting the temperature dependence of SVP35 and SVP70 activity.

Figure 4 is a graph exhibiting the temperature stability of SVP35.

Figure 5 depicts the inhibitory activities of various compounds to SVP35 and SVP70 activity.

Figure 6 depicts the sequential change of the conversion of pro MTG to an active form MTG by SVP35 and SVP70 as respective change of the amount of the protein.
Figure 7 (A) and (B) are the graphs which depict the time course of transglutaminase activity, if a pro MTG is reacted with SVP70 and SAM-P45, respectively. (A): SVP70 addition, ●: addition amount of 1/200 relative to substrate, ■: addition amount of 1/500 relative to substrate; (B): SAM-P45 addition, ◆: addition amount of 1/10 relative to substrate, π: addition amount of 1/50 relative to substrate.

Figure 8 (A) and (B) are the graphs which depict the time course of the amount of MTG protein, if a pro MTG is reacted with SVP70 and SAM-P45, respectively. (A): SVP70 addition, ●: addition amount of 1/200 relative to substrate, ■: addition amount of 1/500 relative to substrate; (B): SAM-P45 addition, ◆: addition amount of 1/10 relative to substrate, π: addition amount of 1/50 relative to substrate.

Description of the Preferred Embodiments

In general, it has been known that a secretory protein is translated as a prepeptide or a propropeptide and thereafter its signal peptide ("a pre-part") is cleaved to be converted into a mature peptide or propeptide, and the propeptide is cleaved at the domain referred to as pro-structure to be a mature peptide. As used herein, a pro-structure part of a secretory protein may be simply referred to as "pro-structure". In addition, as used herein, "a signal sequence" refers to the sequence which is located at the N-terminal of a secretory protein precursor and which is not present in a naturally occurring mature protein, and "a signal peptide" refers to the peptide which is cleaved from such a protein precursor. Generally, a signal sequence is cleaved by a protease following the extracellular secretion.

As used herein, a protein which do not contain a signal peptide but dose contain a pro-structure part may be referred to as "proprotein", for example "protransglutaminase" or "pro MTG". As used herein, a pro-structure part of a secretory protein may be simply referred to as "a pro-structure" or "a pro-structure part", and these terms can be herein used interchangeably.

Among proteases which are assumed to be able to express easily in a coryneform bacterium, the inventors firstly performed the search for a protease having high specificity and selectivity for the substrate of interest, i.e. a protease which selectively cleaves the pro-structure part of a pro MTG, and causes the over-decomposition of the
transglutaminase itself as little as possible.

When MTG is secreted extracellularly by an actinomycetes, it has been assumed to be firstly secreted as a pro MTG, followed by the cleavage of the pro-structure part of the pro MTG resulting in an active form MTG (Eur. J. Biochem., vol. 257, p. 570-576 (1998)). Accordingly, the inventors have expected that a protease that cleaves the pro-structure part of a pro MTG is present within MTG producing actinomycetes. Since this protease is originally a protease that cleaves the pro-structure part, it is expected that the protease has a high selectivity for substrates and cleaves only the pro-structure part, while it acts on the MTG itself in less degree.

In addition, both a structural gene of a pro MTG of actinomycetes and a structural gene of the protease SAM-P45 can be effectively expressed in a coryneform bacterium, and they can be secreted extracellularly. Based on this information, an earnest investigation has been made in order to find the protease of interest from a MTG producing bacterium which is an actinomycetes, and as a result, it was revealed that the MTG producing strain *Streptoverticillium mobaraense* had a high cleavage selectivity for the pro-structure part of the pro MTG and produces new neutral metallopeptases useful for the production of an active MTG. The present inventors isolated and purified these neutral metallopeptases and demonstrated their enzymological properties. Furthermore, the inventors determined the amino acid sequences of the N-terminal parts of these metallopeptases, and obtained the genes encoding the metallopeptases.

In addition, the inventors introduced the enzyme gene into a coryneform bacterium, allowing the expression of it in a system using a coryneform bacterium as a host, and as a result, the enzyme was secreted extracellularly. Furthermore, the enzyme was practically reacted on a pro MTG of a pro-structure part, resulting in the cleavage of the pro-structure part to yield an active transglutaminase. Neutral metallopeptases from microorganisms of other sources having an equivalent function have also found, which have been revealed to be similarly useful for the production of an active form MTG.

The more specific embodiments of the present invention will be illustrated hereinafter.

The neutral metallopeptases according to the present invention can be prepared
from the surfaces of a cultured actinomycetes or culture supernatant of the actinomycetes including *Streptoverticillium mobaraense*, *Streptomyces griseus*, *Streptomyces coelicolor*, etc.

In the following part, the newly found neutral metalloproteases of *Streptoverticillium mobaraense* IFO13819 are firstly described.

The cultivation of a bacterium to obtain the neutral metalloprotease according to the present invention, for example an actinomycetes described above, can be carried out according to the methods conventionally used for the cultivation of actinomycetes. Namely, a common medium containing conventional carbon sources, nitrogen sources, inorganic ions and others can be used as a medium for the culture. Glucose, starch, sucrose and others can be used as the carbon sources. Peptone, yeast extract, meat extract, malt extract, ammonium salt and others are optionally used as the nitrogen sources if necessary. The culture may be conducted under the aerobic condition which is appropriately controlled within the pH range of between pH 5.0 and 8.5 and the temperature range between 15°C and 37°C. For the production of the neutral metalloproteases according to the present invention, the culture is preferably continued until the maximum amount of the intended neutral metalloprotease may be achieved, and then it can be terminated. Although the suitable culture period depends on the temperature, pH and the type of medium, usually the period is preferably about 1 to 12 days. After the culturing period, the culture may be separated into the cells and the culture supernatant by centrifugation and the like.

The new neutral metalloproteases according to the present invention can be obtained from the culture supernatant as well as the recovered cells, in particular from the surface of the cells. For purifying the enzyme, any methods which are conventionally used for purifying a enzyme, for example ammonium sulfate salting-out technique, gel filtration technique, ion-exchange chromatography, hydrophobic chromatography and the like can be adopted. The protease can be purified more efficiently using high performance liquid chromatography (HPLC) etc. The measurement of the enzyme activity of the neutral metalloprotease obtained in this way can be determined by reacting the enzyme with a peptide which contains a region connecting the pro-part of a protransglutaminase and a mature transglutaminase, for example synthetic peptide Glu-Pro-Ser-Phe-Arg-Ala-Pro-
Asp-Ser (SEQ ID NO: 11) (Peptide Institute) as a substrate and calculating the reduced amount of the substrate.

As mentioned above, the neutral metalloprotease according to the invention purified from the recovered cells, in particular from the surface of the cells, or from the supernatant of the culture, can be analyzed for the N-terminal amino acid sequence by a gas phase protein sequencer to determine the partial amino acid sequence. Furthermore, the enzymatic properties (optimum pH, pH stability, optimum temperature, the effect of an inhibitor, etc.) of the isolated and purified neutral metalloprotease can be examined.

In one embodiment of the present invention, the neutral metalloprotease named SVP35 was obtained from the surface of the cells of Streptoverticillium mobaraense and the neutral metalloprotease named SVP70 can be obtained from the culture supernatant of Streptoverticillium mobaraense.

In one embodiment of the present invention, the neutral metalloprotease according to the invention is the neutral metalloprotease SVP35 having the following properties:

1) Molecular weight: about 35,000 (as measured by SDS-PAGE)
2) Optimum pH: 6.0-8.0, more specifically 6.5-7.5, in particular around 7.0
3) pH Stability: pH of 4-10
4) Optimum temperature: about 45°C
5) Temperature stability: it is stable below about 50°C
6) Inhibitors: it is strongly inhibited by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, and by Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes.

In another embodiment of the present invention, the neutral metalloprotease according to the present invention is the neutral metalloprotease SVP70 having the following properties:

1) Molecular weight: about 71,000 (as measured by SDS-PAGE)
2) Optimum pH: 6.0-8.0, more specifically 6.5-7.5, in particular around 7.0
3) pH Stability: pH of 5-10
4) Optimum temperature: about 50°C-55°C, in particular around 55°C
5) Inhibitors: it is strongly inhibited by ethylene diamine tetraacetic acid, 1,10-
phenanthroline and phosphoramidon which are metalloprotease inhibitors, dithiothreitol which is a SH-reductant, and by Streptomyces subtilisin inhibitor (SSI) from actinomycetes.

When SVP35 or SVP70 is reacted on pro MTG, both of them show a high selective cleavage activity on the pro-structure part of the MTG. Namely, since both of the enzymes are characterized by converting the pro MTG into the active MTG efficiently, while the activity for degrading the resulting active MTG itself is low, both of them are the suitable enzymes for producing an active MTG using pro MTG as a raw material. The N-terminal amino acid sequences of the two new neutral metalloproteases are shown in SEQ ID NO: 1 for SVP35, and in SEQ ID NO: 2 for SVP70, which reveals the homology between these sequences. Therefore, those having any homology with these proteases in their N-terminal amino acid sequences were searched and a metalloprotease SGMP II (J. Biochem. Vol. 110, p. 339-344 (1991)) from Streptomyces griseus as well as the three metalloproteases (GenBank/EMBL/DDBJ CAB76000, CAB76001, CAB69762) from Streptomyces coelicolor and the like were found. These proteases can be also used in the same way as SVP35 and SVP70 for the selective cleavage of the pro-structure part of a pro MTG, and they can be used in order to produce an active MTG using a pro MTG as a raw material.

Next, a method of producing the neutral metalloprotease according to the present invention by recombinant DNA technique will be described.

A number of examples of producing useful proteins including enzymes, physiologically active substances and the like using the recombinant DNA technique have been known. The advantage of using the recombinant DNA technique is the ability of mass-production of useful proteins that exist in minute quantity in nature.

For producing the neutral metalloprotease according to the present invention by using the recombinant DNA technique, a genetic construct is firstly generated, where the genetic construct contains a promoter, a sequence encoding a proper signal peptide, a nucleic acid fragment encoding the neutral metalloprotease according to the invention, and a regulatory sequence (an operator or terminator, etc.) which is necessary to express the gene for the neutral metalloprotease in a coryneform bacterium, in a proper position such that they can function. The neutral metalloprotease according to the invention may have a pro-structure part at the N-terminal. Vectors, which can be used for this construct, are not
particularly limited and include any one which can function in a coryneform bacterium, and
they may be those which autonomously replicate such as plasmids or those which are
integrated into the chromosome of the bacterium. When a coryneform bacterium is used as
a host, plasmids derived from coryneform bacteria are particularly preferable as vectors.
These include, for example, pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)),
drug-resistant genes.

Examples of Corynebacterium which can be used as a host bacterium in the
present invention include mutant strains derived from wild type strains including
*Brevisibacterium saccharolyticum* ATCC14066, *Brevisibacterium immariophilum* ATCC14068,
*Brevisibacterium lactofermentum* (Corynebacterium glutamicum) ATCC13869,
*Brevisibacterium roseum* ATCC13825, *Brevisibacterium flavum* (Corynebacterium
*glutamicum*) ATCC14067, *Corynebacterium acetocidofilum* ATCC13870,
*Corynebacterium glutamicum* ATCC13032, *Corynebacterium lilium* (Corynebacterium
*glutamicum*) ATCC15990, *Brevisibacterium ammoniagenes* (Corynebacterium
*ammoniagenes*) ATCC6871 and the like, or mutant strains derived from mutants strain of
these wild types.

Mutant strains which are used in the present invention include, for example mutant
strains defective in the ability to produce glutamate, mutant strains for amino acids
production such as lysine and the like, and mutant strains modified to produce other
substances such as nucleic acids, for example, inosine. Such mutant strains can be
obtained by performing the treatment with ultraviolet irradiation or chemical mutagen such
as N-methyl-N' -nitrosoguanidine and the like, and then selecting the strains of which ability
to secrete-produce proteins has been increased.

Especially, *Corynebacterium glutamicum* AJ1203 (FERM BP-734) (the original
deposition on March 26, 1984) (currently, National Institute of Advanced Industrial Science
and Technology, International Patent Organism Depositary, Tsukuba Central 6, 1-1,
Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan), which has isolated from the
wild type *Corynebacterium glutamicum* (*C. glutamicum*) ATCC13869 as a streptomycin-
resistant mutant strain, is expected to have a mutation in a functional gene associated with
the secretion of proteins, and its ability to secrete-produce heterologous proteins is extremely as high as approximately 2- or 3-fold as a accumulated amount as compared with its parent strain (wild strain) under the optimum culture condition, so it is suitable as a host bacterium (see WO 02/081694). In addition, it is preferable to use a strain which was obtained by modifying such a strain as a host such that the strain does no longer produce the cell surface protein, because the purification of the heterologous proteins secreted in the medium will become easier, so it is particularly preferable. Such a modification can be performed by introducing a mutation into the cell surface protein gene on the chromosome or into its expressional regulatory region through mutagenesis or gene recombination techniques.

Examples of promoters from a coryneform bacterium include promoters for the genes of cell surface proteins PS1, PS2, and SlpA, promoters for the genes in biosynthetic systems of various amino acids, for example, glutamine synthetase gene, aspartokinase gene in the lysine biosynthetic system and the like.

The signal peptide which is used in the present invention is the signal peptide of a secretory protein from coryneform bacterium, the host, and preferably it is the signal peptide of a cell surface protein from a coryneform bacterium. The cell surface proteins of coryneform bacteria include PS1 and PS2 from *C. glutamicum* (JP-Kokai No. 6-502548), and SlpA from *C. ammoniagenes* (JP-Kokai No. 10-108675).

For producing a neutral metalloprotease whose activity for selective cleavage of a pro-structure of a pro MTG is strong by using the recombinant DNA technique, a DNA encoding such a neutral metalloprotease is required.

In one embodiment of the present invention, the neutral metalloprotease SVP35 is produced by using the recombinant DNA technique. The DNA encoding SVP35 can be obtained as follows.

Firstly, the amino acid sequence of the purified SVP35 is determined. Edman method (Edman, P., Acta Chem. Scand. 4, 227 (1950)) can be used to determine the amino acid sequence. Gas-phase protein sequencer from Shimadzu Co. Ltd. Co. Ltd. and the like can be also used to determine the amino acid sequence.

For the neutral metalloprotease SVC35 according to the present invention, the
sequence shown in SEQ ID NO: 1 has been found by sequencing 20 amino acid residues from its N-terminal.

This information can be used to synthesize an appropriate primer for PCR and generate a probe to obtain the neutral metalloprotease according to the present invention. For example, a protease gene from actinomycetes, which is expected to have a homology based on the search results for the homology in the N-terminal amino acid sequence, for example, a metalloprotease (GenBank/EMBL/DDBJ CAB76001) gene from Streptomyces coelicolor can be subjected to PCR using an actinomycetes DNA prepared by the method of Saito and Miura [Biochem. Biophys. Acta, 72, 619 (1963)] as a template, to amplify the fragment of the gene encoding this protease. The amplified fragment can be used as a probe.

Then, the actinomycetes DNA prepared by the method of Saito and Miura, for example the chromosomal DNA of Streptoverticillium mobaraense IFO13819, is digested with appropriate different restriction enzymes, for example various restriction enzymes which recognize 6-base sequences. The digested actinomycetes chromosomal DNA can be analyzed by the techniques well known to those skilled in the art such as Southern blot hybridization technique described in Molecular Cloning 2nd edition [J. Sambrook E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p9. 31 (1989)] and the like by using the $^{32}$P-labeled PCR-product obtained by the above-described PCR. For example, the nucleic acid molecule encoding the neutral metalloprotease according to the present invention or the part thereof can be cloned by recovering the fragment which has been confirmed by Southern blot to have a high homology with the used probe, and cloning it into an appropriate vector. The techniques necessary for such a gene cloning are well known to those skilled in the art (see for example, J. Sambrook E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p1. 90 (1989)).

In one embodiment of the present invention, PCR is performed using the chromosomal DNA of Streptomyces coelicolor A3(2) as a template to produce a probe. Furthermore, a single band of about 8 kb hybridizable with $^{32}$P-labeled probes detected in the digested product of Streptoverticillium mobaraense IFO13819 chromosomal DNA digested with SphI. Thus, the chromosomal DNA of Streptoverticillium mobaraense
IFO13819 prepared by the foregoing method is digested with SphI, the fragment of about 8 kb is recovered through an agarose gel electrophoresis, the recovered fragment is introduced at the SphI site in pUC18, and then it is introduced into a competent cell of *Escherichia coli* JM109 to generate a library. The clones of interest can be obtained by screening the generated library using a synthetic oligonucleotide as a probe according to the colony hybridization techniques described in Molecular Cloning 2nd edition (supra), and selecting the strain which harbors the plasmid containing the gene fragment of SVP35 cloned into the plasmid. The plasmid recovered from this strain is herein designated as pVSV1. The nucleotide sequence of the fragment cloned into pVSV1 is analyzed, the primary amino acid sequence is deduced to confirm that the fragment encodes the previously determined N-terminal amino acid sequence. Thus, the obtained gene is confirmed to be the gene encoding SVP35.

Then, a recombinant nucleic acid molecule can be constructed to express the neutral metalloprotease according to the present invention by ligating a genetic construct containing the DNA encoding the obtained metalloprotease to an appropriate vector depending on the properties of the host used. The host, coryneform bacterium cells are transformed with the recombinant nucleic acid molecule. The transformed cells can be cultured in a suitable medium to recover the neutral metalloprotease according to the present invention secreted or accumulated in the medium and/or in the cell.

Next, a method of producing an active MTG from pro MTG using the neutral metalloprotease will be described.

The neutral metalloprotease used in the production of an active MTG can be reacted on a pro MTG as a fraction containing the neutral metalloprotease prepared from the culture medium of a neutral metalloprotease producing bacterium. It can be also used as more highly purified neutral metalloprotease with high specific activity. Furthermore, as described below, the neutral metalloprotease can be also used, wherein the neutral metalloprotease can be obtained by culturing the cell transformed with a recombinant nucleic acid molecule which may be obtained by connecting a DNA encoding a neutral metalloprotease having strong selective cleavage activity for a pro-structure part of pro MTG.
The pro MTG used for the production of MTG may be a fraction containing the pro MTG prepared from the culture medium a pro MTG producing bacterium. More highly purified pro MTG may be also used. The reaction may be performed under the condition that the amount of a neutral metalloprotease added to the pro MTG is from 1/10 to 1/500 by weight and that is appropriately adjusted within the reaction temperature ranging between 15°C and 50°C and the pH range of between pH 5.0 and 9.

In addition, the genetic construct, which is constructed as described above and which contains the DNA encoding the neutral metalloprotease according to the present invention, may be introduced into a microorganism containing the genetic construct encoding a pro MTG, in particular, into a coryneform bacterium, to produce in a single bacterial cell both of the pro MTG and the neutral metalloprotease according to the present invention, thereby the pro MTG may be converted into a mature MTG under the above condition. More detailed method for efficiently producing a pro MTG in coryneform cells, the genetic construct used for such a method, and a coryneform bacterium into which the genetic construct has been introduced are disclosed in, for example WO 01/23591. More specifically, for example, a coryneform bacterium which can efficiently secrete a pro MTG protein extracellularly may be obtained by introducing a genetic construct into a coryneform bacterium, wherein the genetic construct is obtained by connecting the sequence having the sequence encoding a pro MTG, which is located downstream to the sequence encoding the signal peptide domain of a coryneform bacterium, particularly the signal peptide domain of a cell surface protein, downstream to an appropriate promoter. The signal peptide, promoter and host which can be used for this purpose can be selected from signal peptides, promoters and hosts which are suitable for expressing the neutral metalloproteases according to the present invention as above mentioned. A combination of vectors that are compatible in the same cell is also well known to those skilled in the art. Therefore, the mature MTG can be obtained by introducing an appropriate genetic expression construct containing the DNA encoding the neutral metalloprotease according to the present invention as mentioned above into a coryneform bacterium producing a pro MTG, or vice versa, by introducing an appropriate genetic expression construct encoding a pro MTG into coryneform bacterium producing the neutral metalloprotease according to the present
invention, thereby allowing the genetic constructs which can express the pro MTG and the neutral metalloprotease according to the present invention to coexist in the same bacterium, culturing the bacterium, and keeping the culture in an appropriate condition such that the neutral metalloprotease according to the present invention may be active.

The transglutaminase produced by the method according to the present method can be isolated and purified from reaction mixture according to the methods well known to those skilled in the art. For example, the transglutaminase can be isolated and purified by removing the cells from the mixture by centrifugation, etc. and then by using known appropriate methods such as salting-out, ethanol precipitation, ultrafiltration, gel filtration chromatography, ion-exchange column chromatography, affinity chromatography, medium high-pressure liquid chromatography, reversed-phase chromatography, hydrophobic chromatography or the combination thereof.

The present invention is further described in the following Examples, which should not be construed in any way as the limitation of the present invention.

Examples

Example 1: Neutral metalloprotease produced by Streptoverticillium mobaraense IFO13819

(1) Purification of Neutral metalloprotease (SVP70) produced by Streptoverticillium mobaraense IFO13819

5 L of Sakaguchi flask (shaking flask) was received 800 mL of ISP2 culture medium (4 g of Yeast Extract, 10 g of Malt Extract, 4 g of Glucose per liter of water, adjusted to pH 7.3), and was inoculated with Streptoverticillium mobaraense IFO13819 from a plate, and cultured by shaking at 30°C for 9 days at 120 rpm. The culture medium was centrifuged, and the supernatant of the culture was collected. It was filtered using Depth filter (3 μm of pore size, Salt torius Co. Ltd.), followed by concentration using Sartocon Slice membrane having a pore size of 10,000Da (Salt torius Co. Ltd.). The concentrate was diluted 10-fold with Tris-HCl buffer / 5mM calcium chloride (pH 7.5), subjected to DEAE-Sepharose FF (2.6 cm x 10 cm, Amersham Pharmacia Co. Ltd.) column equilibrated with the same buffer, using FPLC (Amersham Pharmacia Co. Ltd.), and eluted using linear concentration
gradient of 0-0.5 M sodium chloride. A fraction containing active ingredient was collected, subjected to phenyl Sepharose HP (1.6 φ x 10 cm, Amersham Pharmacia Co. Ltd.) column equilibrated with 1.5 M ammonium sulfate / 20 mM MES buffer / 5 mM calcium chloride (pH 6.0), eluted using linear concentration gradient of 1.5-0 M ammonium sulfate, and an active fraction was collected. The resulting active fraction was dialyzed against 20 mM MES buffer / 5 mM calcium chloride (pH 6.0) at 4°C overnight to obtain a purified enzyme solution.

The measurement of the enzyme activity at each step was carried as follows:

The enzyme solution was added to 20 mM sodium phosphate buffer containing peptide GPSFRAPDS (Peptide Institute) (SEQ ID NO: 11) to yield 170 μl of total liquid volume, and it was reacted at 30°C for 10 minutes, followed by heating at 95°C for 5 minutes to terminate the reaction. 80 μl of this solution was analyzed by HPLC under the following condition and its activity was calculated based on the decreased amount of the substrate.

Apparatus : HPLC L-6300 system (Hitachi Co. Ltd).
Column : YMC-PACK ODS 120A 4.6 x 150 mm (YMC)
Eluent : (A) 0.1% TFA  (B) 80% acetonitrile / 0.1% TFA
Eluting condition : linear concentration gradient of 12-16% acetonitrile (for 15 minutes)
Flow rate : 1.0 ml / min
Detection wavelength : 220 nm

Under this condition, the peptide GPSFRAPDS was eluted from 13 to 14 minutes of retention time, and the degraded product FRAPDS was eluted from 7.5 to 8.5 minutes of retention time.

The amount of the enzyme that catalyzes one (1) nmol of pro MTG decomposition in a minute was defined as one (1) unit of the enzyme activity.

(2) Purification of Neutral metalloprotease (SVP35) produced by Streptoverticillium mobaraense IFO13819

5 L of Sakaguchi flask was received 800 mL of ISP2 culture medium and was
inoculated with *Streptovercillium mobaraense* IFO13819 from a plate, and cultured by shaking at 30°C for 48 hours at 120 rpm. The culture medium was centrifuged, and the supernatant of the culture was discarded to harvest cells. The cells were suspended in 20 mM Tris-HCl buffer / 30 mM sodium chloride (pH 7.5), shaken on ice for 4 hours, and then the supernatant was collected by centrifugation. The supernatant obtained was filtered and sterilized using Depth filter (0.22 μm of pore size, made by Saltorius Co. Ltd.), and then it was subjected to CM-Sepharose FF (Amersham Pharmacia Co. Ltd.) column (1.6 φ x 10 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM calcium chloride and 0.01 mM zinc chloride, using FPLC (Amersham Pharmacia Co. Ltd.), eluted in the same buffer using linear concentration gradient of 0-0.5 M sodium chloride. A fraction containing active ingredient was collected, and was further subjected to Phenyl-Sepharose HP column (1 mL, Amersham Pharmacia Co. Ltd.) equilibrated with 20 mM Tris-HCl buffer containing 1.5 M ammonium sulfate, 5 mM calcium chloride and 0.01 mM zinc chloride, and eluted using linear concentration gradient of 1.5-0 M ammonium sulfate. An active fraction was collected, and demineralized by 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM calcium chloride and 0.01 mM zinc chloride, using PD-10 column (Amersham Pharmacia) to give a part purified enzyme solution.

The enzyme activity at each step was measured using peptide GPSFRAPDS as a substrate in the same manner as in (1).

(3) Evaluation of the properties of the neutral metalloprotease (SVP35) produced by *Streptovercillium mobaraense* IFO13819

i) Substrate specificity

1 mg/ml of insulin B solution and pro MTG solution prepared in 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM calcium chloride and 0.01 mM zinc chloride was used as a substrate, and an enzyme solution was added into the solution to react at 30°C for 2 hours, and then peptide fragments were separated by HPLC under the following condition:

**Apparatus:** L-7100 / 7200 / 7405 / D-7600 (Hitachi Co. Ltd.)

**Column:** VYDAC C18 4.6 mm l.D. x 250 mm (VYDAC)
Eluent: (A) 0.1% TFA (B) 80% acetonitrile / 0.1% TFA
Eluting condition: linear concentration gradient of 4-44% acetonitrile
Flow rate: 0.5 ml/min
Detection wavelength: UV 220 nm

The amino acid sequences of the obtained peptide fragments were analyzed by PPSQ-10 (Shimadzu Co. Ltd.) to characterize the sequences of the cleavage points for SVP35. As a result, it was confirmed that the peptide was cleaved before (at the N-terminal of) especially Phe, often Leu, sometimes Tyr, Trp, Ile, Val, and that SVP recognized the aromatic amino acids and hydrophobic amino acids with bulky side-chains positioned at P' 1 of the cleavage site.

ii) Optimum pH

In 0.15 M GTA buffer (buffered by 3,3-dimethyl glutaric acid, Tris (hydroxy methyl) amino methane, 2-amino-2-methyl-1,3-propanediol) from pH 3 to pH 10, SVP35 was allowed to act on Gly-Pro-Ser-Phe-Arg-Ala-Pro-Asp-Ser as a substrate at 30°C for 10 minutes. As a result, it was revealed that the Optimum pH of SVP35 was around 7.0, and that when the activity at pH 7.0 was defined as 100%, SVP35 had an activity of 70% or more at pH 6.0-8.0 and an activity of 80% or more at pH 6.5-7.5 (see Figure 1).

iii) pH stability

To 10 µl of SVP35 purified enzyme solution, 40 µl of each pH of 0.15 M GTA buffer from pH 3 to pH 10 was added, left at 4°C overnight, followed by addition of 0.1 M sodium phosphate buffer (pH 7.0) to fit the liquid volume to 400 µl, and was adjusted to pH 7.0. To these enzyme solutions, Gly-Pro-Ser-Phe-Arg-Ala-Pro-Asp-Ser was added as a substrate, and reacted at pH 7.0, at 30°C for 10 minutes. As a result, it was shown that SVP35 was stable within the range of pH 4 to pH 10 (when the activity at pH 4.0 was defined as 100%, it had an activity of 90% or more at pH 4-10) (see Figure 2).

iv) Optimum temperature

To the purified enzyme solution diluted with 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM calcium chloride and 0.01 mM zinc chloride Gly-Pro-Ser-Phe-Arg-Ala-Pro-Asp-Ser was added and reacted at pH 7.0, between 5°C and 65°C for 10 minutes. As a
result, it was shown that the Optimum temperature of SVP35 was about 45°C and it had high activity within the range of 40°C to 50°C (it had an activity of 80% or more than that at 45°C) (see Figure 3).

v) Temperature stability

To 10 µl of the purified enzyme solution, 40 µl of 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM calcium chloride and 0.01 mM zinc chloride was added to treat at 4°C or at from 30°C to 70°C for 15 minutes, and then cooled by ice, added 250 µl of 20 mM sodium phosphate buffer (pH 7.0). To this enzyme solution, Gly-Pro-Ser-Phe-Arg-Ala-Pro-Asp-Ser was added as a substrate and reacted at 30°C for 5 minutes. When the activity treated at 4°C was defined as 100%, the remaining activity at each temperature was calculated. As a result, it was shown that SVP35 retained 80% of activity at 50°C, but it lost its activity at 60°C (see Figure 4).

vi) Inhibitors

To 20 mM sodium phosphate buffer (pH 7.0) containing various compounds of the concentration shown in Figure 5, the purified enzyme solution was added and left for 60 minutes at room temperature. Then Gly-Pro-Ser-Phe-Arg-Ala-Pro-Asp-Ser was added as a substrate and reacted for 10 minute at 30°C. The relative activity by adding each compound was calculated based on the Gly-Pro-Ser-Phe-Arg-Ala-Pro-Asp-Ser cleavage activity in the absence of compounds as100%. As a result, it was shown that SVP35 was strongly inhibited by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, and by Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes (see Figure 5).

(4) Characterization of the properties of the neutral metalloprotease (SVP70) produced by Streptoverticillium mobaraense IFO13819

i) Substrate specificity

The substrate specificity was examined similarly as described in (3)-i). As a result, it was revealed that the substrate was cleaved before (at N-terminal side of ) especially Phe, often Leu, sometimes Tyr, Trp, Ile, Val, and that SVP70 recognized the aromatic amino acids and hydrophobic amino acids with bulky side-chains positioned at P' 1 of the
cleavage site.

ii) Optimum pH

The Optimum pH of SVP70 was examined similarly as (3)-ii). As a result, it was revealed that the Optimum pH of SVP70 was around 7.0, and that if the activity at pH 7.0 is defined as 100%, SVP70 had an activity of 90% or more at pH 6.0-8.0 and an activity of 95% or more at pH 6.5-7.5 (see Figure 1).

iii) pH stability

The pH stability was examined similarly to (3)-iii). As a result, it was shown that SVP70 was stable within pH 5 to pH 10, but it was less stable than SVP35 at slightly alkaline (see Figure 2). Specifically, if the activity at pH 5.0 was defined as 100%, it had an activity 90% or more in the range of pH 5 to pH 7, and it had an activity about 80% or more even in the range of pH 7 to pH 10.

iv) Optimum temperature

The Optimum temperature of SVP70 was examined similarly as (3)-iv). As a result, it was shown that the Optimum temperature of SVP70 was within the range from about 50°C to 55°C, especially around 55°C (see Figure 3).

v) Inhibitors

The inhibitory activities of various compounds to SVP70 were examined analogously to (3)-vi). As a result, SVP70 underwent strong inhibitory action by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon, which are metalloprotease inhibitors, and by reductant dithiothreitol, urea, and Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes (see Figure 5).

(5) Sequencing of the N-terminal amino acid sequence of SVP35 and SVP70

The purified enzymes of SVP35 and SVP70 obtained in (1) and (2) above were transferred onto Polyvinilidene-difluoride (PVDF) membrane using Membrane Cartridge (Perkin Elmer Co. Ltd.) and N-terminal amino acid sequence was analyzed using gas-phase Protein Sequencer PPSQ-10 (Shimadzu Co. Ltd.). The amino acid sequence of SVP35 is shown in SEQ ID NO: 1, and the amino acid sequence of SVP70 is shown in SEQ ID NO: 2. A homology can be seen in these sequences.
Accordingly, those which had any homology to these proteases for N-terminal amino acid sequences were searched, and then metalloprotease SGMP II (J. Biochem., Vol. 110, p. 339-344, 1991) from *Streptomyces griseus*, and three metalloproteases (GenBank/EMBL/DDBJ CAB76000, the same CAB76001, and the same CAB69762), etc. from *Streptomyces coelicolor* were found. These proteases also can be used to cleave selectively the pro-structure part of the pro MTG, and therefore they can be used to produce an active form MTG according to the prevent invention.

(6) Cloning of SVP35 gene and its secretory expression in coryneform bacteria

The chromosomal DNA of *Streptomyces coelicolor* A3(2) was prepared using the method of Saito and Miura [Biochem. Biohys. Acta, 72, 619 (1963)]. Primers shown in SEQ ID NO: 3 and SEQ ID NO: 4 were synthesized on referring to the sequence of metalloprotease (GenBank/EMBL/DDBJ CAB76001) gene from *Streptomyces coelicolor* which have a homology in the N-terminal amino acid sequence. Primers shown in SEQ ID NO: 3 and SEQ ID NO: 4 were used to perform PCR using the chromosomal DNA of *Streptomyces coelicolor* A3(2) as a template, and the gene region in the metalloprotease gene was amplified. For PCR reaction, Pyrobest DNA polymerase (Takarasyuzo Co. LTD.) was used and the reaction condition followed the protocol recommended by the manufacturer. The chromosomal DNA of *Streptoverticillium mobaraense* IFO13819 prepared by the method of Saito and Miura was digested by various restriction enzymes which recognize 6-base sequence, the digested samples were analyzed by Southern blot hybridization as described in Molecular Cloning 2nd edition [J. Sambrook E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p. 9. 31 (1989)], using the $^{32}$P-labeled PCR product as a probe, and a single band of about 8 kb was detected by SphI cleavage.

Accordingly, the chromosomal DNA of *Streptoverticillium mobaraense* IFO13819 which had been prepared by the forgoing method was digested with SphI, and the fragment of about 8 kb was recovered through agarose gel electrophoresis using EASYTRAP Ver. 2 (Takarasyuzo Co. LTD.). The recovered fragment was inserted into SphI site of pUC18, which was introduced into competent cells of *Escherichia coli* JM109 (Takarasyuzo Co. LTD.) to generate a library. The library was screened for the bacterium strain which
contains the plasmid where the SVP35 gene fragment was cloned, by colony hybridization as described in Molecular Cloning 2nd edition [J. Sambrook E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p1. 90 (1989)], using the synthetic nucleotide as a probe.

The plasmid was recovered from the strain obtained above and was designated as pVSV1. The nucleotide sequence of the fragment cloned in pVSV1 was determined. The nucleotide sequence of this cloned fragment is shown in SEQ ID NO: 5. The primary amino acid sequence encoded by this gene was deduced, which allowed the determination of the entire primary amino acid sequence of SVP35 containing the signal sequence of SVP35 including the amino acid sequence of previously determined N-terminal portion and the region assumed as a pro-structure part. The entire amino acid sequence of SVP35 is shown in SEQ ID NO: 6. It is presumed that amino acids nos. 1-36 of amino acid sequence described in SEQ ID NO: 6 refer to the signal sequence, amino acids nos. 37-216 refer to the pro-structure part, and amino acids nos. 217-537 correspond to the mature SVP35.

Primers shown in SEQ ID NO: 7 and SEQ ID NO: 8 were synthesized using pVSV1 as a template on referring to the sequence of SEQ ID NO: 5, and the gene region containing the pro-structure part of SVP35 and the mature SVP35 was amplified by PCR technique. For PCR reaction, Pyrobest DNA polymerase (Takarasyuzo Co. Ltd.) was used and the reaction condition followed the protocol recommended by the manufacturer.

Next, using pPKSPTG1 described in WO 01/23591 as a template, the region including 5'-upstream region containing the promoter region of PS2 gene which is cell surface protein of C. glutamicum and the signal sequence of SlpA, cell surface protein of C.ammoniagenes was amplified by PCR technique using the combination of oligonucleotides of SEQ ID NO: 9 and SEQ ID NO: 10. The primer shown in SEQ ID NO: 10 contains the sequence encoding the N-terminal amino acids of SVP35 having a pro-structure part.

Then, the gene of the heterologous fusion pre-pro SVP35 gene fragment, which was connected to the 5'-upstream region comprising the promoter region of PS2 gene and the signal sequence of SlpA, the cell surface protein, from C.ammoniagenes, was amplified by performing cross-over PCR with SEQ ID NO: 8 and SEQ ID NO: 9 using the mixture of 1
µl of each of the amplified PCR solution. The amplified fragment of about 2.3 kb was
detected by agarose gel electrophoresis. The PCR product was subjected to agarose gel
electrophoresis to recover a fragment of about 2.3 kb, and after blunting its ends using DNA
Blunting Kit (Takarasyuzo Co. Ltd.), the fragment was inserted into Smal site of pCV7 as
described in JP-Kokai No. 9-070291 to obtain pVSV1. The nucleotide sequence of the
inserted fragment was determined according to the conventional method to confirm that the
fusion gene was constructed as expected.

*C. glutamicum* ATCC13869 was transformed with the constructed pVSV1 and the
strains grown on the CM2S agar medium comprising 5 mg/l of chloramphenicol (10 g of
yeast extract, 10 g of tryptone, 5 g of sucrose, 5 g of NaCl, 15 g of agar per liter of distilled
water) were selected. Then, the selected *C. glutamicum* ATCC13869 harboring pVSV1 was
cultured in MMTG culture medium (60 g of glucose, 0.4 g of magnesium sulfate
heptahydrate, 30 g of ammonium sulfate, 1 g of potassium dihydrogenphosphate, 0.01 g of
ferrous sulfate heptahydrate, 0.01 g of manganese sulfate pentahydrate, 450 µg of
thiamine hydrochloride, 450 µg of biotin, 0.15 g of DL-methionine, 50 g of calcium
carbonate per liter of distilled water, adjusted to pH 7.5) comprising 5 mg/l of
chloramphenicol at 30 °C for 30 hours. 1ml of the culture medium was centrifuged to
separate to the supernatant of the culture and the bacteria. The activity of SVP35 was
detected in the supernatant of the culture, and as a result of SDS-PAGE (*Nature*, 227,
380-685 (1970)) electrophoresis according to Laemml's method, it was confirmed that
about 200 mg/L of SVP35 was secretory-expressed.

**Example 2: Conversion of transglutaminase produced by Streptoverticillum mobaraense
IFO13819 precursor (pro MTG) into an active form**

Using pro MTG (1 mg/ml) expressed by *Corynebacterium glutamicum* as a purified
substrate, the neutral protease (SVP35, SVP70) from *Streptoverticillum mobaraense* or the
neutral metalloprotease SGMP II from *Streptomyces griseus* was mixed in the ratio of the
substrate : the enzyme = 200 : 1, the mixture was reacted at 30 °C. After 0, 1, 2, 4, 7, 20
hours, the reaction mixture was sequentially picked up, and the aliquots of the reaction
mixture were mixed with SDS-PAGE sample buffer and heated at 95 °C for 3 minutes, and
then subjected to SDS-PAGE according to Laemmli’s method (Nature, 227, 680-685 (1970)). The result is shown in Figure 6. As can be seen in Figure 6, when these proteases were reacted, pro MTGs were converted to the mature forms, and the produced MTGs were not reduced even after a long-term reaction. The transglutaminase (TG) activity of the picked up fraction was measured by hydroxamate method, and the sufficient activity was confirmed. In addition, SGMP II was purified from actinase (Kakenseiyaku Co. Ltd.) according to the reference method (J. Biocem., Vol. 110, p. 339-344, 1991).

Then, the neutral metalloprotease SVP70 from Streptoverticillium mobaraense, and serine protease SAM-P45 (Streptomyces albogriseolus) as a control, were added to the pro MTGs with gradually increasing the amount of these enzymes, and reacted at 30°C and pH 7.0. After 1, 4, 7, and 24 hours, the reaction mixture was picked up sequentially to determine the TG activity by the hydroxamate method (see Figure 7). The protein concentration of TG was measured by reverse phase chromatography (see Figure 8). As a result, it was shown that SVP could convert pro MTG to the active MTG with an amount as small as 1/500 of the substrate. It was shown that SAM-P45 generated only insufficient transglutaminase activity even at an amount of 1/50 of the substrate, and that the complete conversion to the active form was not observed. On the other hand, when SAM-P45 was added at an amount of 1/10 of the substrate, the conversion into the active MTG was observed, but the decrease in the amount and the activity of MTG-protein were observed. This suggests that over-decomposition of the mature MTG occurred by SAM-P45.

The present invention provides a new protease from an actinomycetes, Streptoverticillium mobaraense, which specifically cleaves the pro-structure part of transglutaminase precursor to activate it, and the gene thereof. The new protease according to the present invention can be expressed in a large amount by a coryneform bacterium, and thereby the present invention provides a method for producing transglutaminase from microorganisms efficiently.

The advantage of using the neutral metalloproteases from actinomycetes according to the present invention for the production of an active MTG is that these enzymes have a strong activities of selectively cleaving the pro-structure part of the pro
MTG, and that these enzymes can be expressed extracellularly by a Coryneform bacterium.

As it is shown that the pro MTG from actinomycetes can be efficiently expressed and secreted by a Coryneform bacterium, it is possible to produce more efficiently an active MTG by a single bacterial cell by co-expressing and secreting the pro MTG and the neutral metalloprotease. In this instance, it is sufficient to express the neutral metalloprotease in only an amount required and sufficient for cleaving the pro-structure part of the pro MTG.
References

1. JP-Kokoku No. 1-50382
2. JP-Kokai No. 64-27471
3. WO publication No. 01/2351
4. JP-Kokai No. 6-502548
5. JP-Kokai No. 10-108675
SEQUENCE LISTING

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Lys Lys Ser Ala Leu Ser Ala Ala Ala Ala Asp Gin Lys Thr Ala Lys Ala
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Glu Ser Gly Gly Leu Asn Glu Ala Thr Ser Asp Ile Phe Gly Thr Ala
370 375 380

Val Glu Phe Tyr Ala Asn Asn Lys Thr Asp Val Gly Asp Tyr Leu Ile
385 390 395 400

Gly Glu Lys Ile Asn Ile Tyr Gly Asp Gly Lys Pro Leu Arg Tyr Met
405 410 415

Asp Lys Pro Ser Lys Asp Gly Lys Ser Lys Asp Ser Trp Tyr Ser Gly
420 425 430

Ile Gly Gly Val Val His Tyr Ser Ser Gly Pro Ala Asn His Phe
435 440 445

Phe Tyr Leu Leu Ser Glu Gly Ser Gly Lys Thr Ile Asn Gly Val
450 455 460

Asp Tyr Asp Ser Pro Thr Ala Asp Gly Ser Lys Val Thr Gly Ile Gly
465 470 475 480

Arg Asp Lys Ala Gln Lys Ile Trp Tyr Lys Ala Leu Thr Thr Gln Phe
485 490 495

Thr Ser Asn Thr Asn Tyr Ala Lys Ala Arg Thr Gly Thr Leu Asn Ala
500 505 510

Ala Ala Ser Leu Tyr Gly Asn Asn Ser Ala Glu Tyr Lys Ala Val Ala
515 520 525

Ala Ala Trp Ser Ala Ile Asn Val Lys
530 535

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<212> DNA
<213> Artificial

<220>
<223> PCR primer
<400> 7
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<210> 8
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<210> 9
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<212> DNA
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<220>
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<400> 9
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<210> 10
<211> 44
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<213> Artificial

<220>
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<400> 10
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<210> 11
<211> 9
<212> PRI
<213> Streptoverticillium mobaraense

<400> 11
Glu Pro Ser Phe Arg Ala Pro Asp Ser
1  5
What is claimed:

1. A method of producing an active microbial transglutaminase from a microbial protransglutaminase, comprising culturing a microorganism into which a gene encoding a neutral metalloprotease from actinomycetes has been introduced, thereby producing the neutral metalloprotease, cleaving a pro-structure part of the protransglutaminase by the neutral metalloprotease which is produced by the microorganism.

2. The method according to claim 1, wherein the microorganism into which a gene encoding a neutral metalloprotease from actinomycetes is a coryneform bacterium.

3. The method according to claim 1, wherein the neutral metalloprotease from actinomycetes is characterized in that
   1) it has a molecular weight of about 35,000;
   2) it has the optimum pH of pH7.0;
   3) it is stable at pH of pH4-10;
   4) it has the optimum temperature of about 45°C;
   5) it is stable below about 50°C; and
   6) it is strongly inhibited by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, and by Streptomyces subtilisin inhibitor (SSI) from actinomycetes.

4. The method according to claim 1, wherein the neutral metalloprotease from actinomycetes is characterized in that
   1) it has a molecular weight of about 71,000;
   2) it has the optimum pH of 7.0;
   3) it is stable at pH of 5-10;
   4) it has the optimum temperature of about 55°C; and
   5) it is strongly inhibited by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, dithiothreitol which is a SH-reductant, and by Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes.
5. A neutral metalloprotease from actinomycetes characterized in that
1) it has a molecular weight of about 35,000;
2) it has the optimum pH of 7.0;
3) it is stable at pH of 4-10;
4) it has the optimum temperature of about 45°C;
5) it is stable below about 50°C; and
6) it is strongly inhibited by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, and by Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes.

6. A neutral metalloprotease from actinomycetes characterized in that
1) it has a molecular weight of about 71,000;
2) it has the optimum pH of 7.0;
3) it is stable at pH of 5-10;
4) it has the optimum temperature of about 55°C; and
5) it is strongly inhibited by ethylene diamine tetraacetic acid, 1,10-phenanthroline and phosphoramidon which are metalloprotease inhibitors, dithiothreitol which is a SH-reductant, and by Streptomyces subtilisin inhibitor (SSI) derived from actinomycetes.

7. A nucleic acid molecule encoding the neutral metalloprotease from actinomycetes according to claim 5 or 6.

8. A method of producing a neutral metalloprotease from actinomycetes, comprising culturing a coryneform bacterium into which the nucleic acid molecule according to claim 7 has been introduced and recovering the neutral metalloprotease from actinomycetes which is secreted extracellularly.
Application number: numéro de demande: **JP/0402923**

Figures: **6**

Pages: 

**DRW-IP**

Unscannable items received with this application

(Request original documents in File Prep. Section on the 10th Floor)

Documents reçus avec cette demande ne pouvant être balayés

(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)
FIG. 3

RELATIVE ACTIVITY (%)

0 10 20 30 40 50 60 70

TEMPERATURE (°C)

- SVP70
- SVP35

FIG. 4

REMAINING ACTIVITY (%)

0 20 40 60 80

TEMPERATURE (°C)
FIG. 5

CONTROL (NONE)
PMSF (5mM)
NEM (5mM)
IODOACETAMIDE (5mM)
LEUPEPTIN (5mM)
EDTA (10mM)
1,10-PHENANTHROLINE (5mM)
PHOSPHORAMIDON (100 μg/ml)
DTT (5mM)
SSI (100 μg/ml)
Urea (4M)

RELATIVE ACTIVITY (%)
FIG. 7

(A) U/ml

- SVP70 (1/200)
- SVP70 (1/500)

(B) U/ml

- SAM-P45 (1/10)
- SAM-P45 (1/50)