(12) STANDARD PATENT (11) Application No. **AU 2005257281 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Method of producing theanine

(51) International Patent Classification(s)
C12P 13/02 (2006.01)

(21) Application No: **2005257281** (22) Date of Filing: **2005.06.22**

(87) WIPO No: **WO06/001296**

(30) Priority Data

(31) Number	(32) Date	(33) Country
2004-189048	**2004.06.28**	**JP**
2004-376443	**2004.12.27**	**JP**

(43) Publication Date: **2006.01.05**
(44) Accepted Journal Date: **2010.08.12**

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(56) Related Art
JP 8-89266
WO 2004/016798
JP 5-68578
JP 5-328986
JP 11-225789

(12) 特許協力条約に基づいて公開された国際出願

(19) 世界知的所有権機関
国際事務局

(43) 国際公開日
2006年1月5日 (05.01.2006)

PCT

(10) 国際公開番号
WO 2006/001296 A1

(51) 国際特許分類[7]: C12P 13/02

(21) 国際出願番号: PCT/JP2005/011420

(22) 国際出願日: 2005年6月22日 (22.06.2005)

(25) 国際出願の言語: 日本語

(26) 国際公開の言語: 日本語

(30) 優先権データ:
特願2004-189048 2004年6月28日 (28.06.2004) JP
特願2004-376443 2004年12月27日 (27.12.2004) JP

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(81) 指定国 (表示のない限り、全ての種類の国内保護が可能): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) 指定国 (表示のない限り、全ての種類の広域保護が可能): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), ユーラシア (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), ヨーロッパ (AT, BE,

[続葉有]

(54) **Title:** METHOD OF PRODUCING THEANINE

(54) 発明の名称: テアニンの製造法

	A 菌の種類		F 実施例1 (Bacillus subtilis)	G 実施例2 (Bacillus amyloliquefaciens)	H 実施例3 (Bacillus coagulans)	I 実施例4 (Bacillus licheniformis)	J 実施例5 (Bacillus cereus)	K 比較例1 (Pseudomonas nitroreducens)
B	培養上清のグルタミナーゼ比活性(mU／mg)		67	53	43	40	5	15
C	L-グルタミンからのモル転換率(%)	D テアニン	75	74	70	69	55	50
		E グルタミン酸	2	3	5	7	10	10

A MICROORGANISM
B SPECIFIC GLUTAMINASE ACTIVITY OF CULTURE SUPERNATANT (mU/mg)
C MOLAR CONVERSION RATIO FROM L-GLUTAMINE (%)
D THEANINE
E GLUTAMIC ACID
F EXAMPLE 1
G EXAMPLE 2
H EXAMPLE 3
I EXAMPLE 4
J EXAMPLE 5
K COMPARATIVE EXAMPLE 1

(57) **Abstract:** It is intended to provide a novel method of efficiently producing theanine so as to enable the convenient and industrially advantageous production of theanine with the formation of little by-product. The above object can be achieved by using a mixture of glutamine with an ethylamine derivative together with glutaminase originating in one or more microorganisms selected from those belonging to the genus *Bacillus*, molds (in particular, those belonging to the genera *Aspergillus, Rizopus* and *Mucor*) and yeasts (in particular, those belonging to the genera *Hansenulla, Saccharomyces* and *Candida*).

(57) 要約: 本発明は、テアニンの効率的な新規製造法を提供し、副産物が少なく、簡易かつ工業的に有利なテアニン生産を可能とすることを目的とする。グルタミンとエチルアミン誘導体の混合物に、Ｂａｃｉｌｌｕｓ属、カビ（特に、Ａｓｐｅｒｇｉｌｌｕｓ属、Ｒｉｚｏｐｕｓ属、Ｍｕｃｏｒ属）、または酵母（特に、Ｈａｎｓｅｎｕｌｌａ属、Ｓａｃｃｈａｒｏｍｙｃｅｓ属、Ｃａｎｄｉｄａ属）由来のうちの一種または二種以上の微生物由来のグルタミナーゼを用いることで上記課題が解決される。

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BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

添付公開書類:
— 国際調査報告書

２文字コード及び他の略語については、定期発行される各*PCT*ガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

METHOD OF MAKING THEANINE

BACKGROUND OF THE INVENTION

1. Field of the invention

The present invention relates to a novel method of making theanine.

2. Description of the related art

Theanine is known as a principal component of deliciousness of green tea and is an important substance as a flavor component of food such as tea. It is pointed out that γ-glutamyl derivative containing theanine acts as a biologically active substance in animals and plants. For example, it is reported that theanine or L-glutamine competes for convulsion caused by caffeine (Chem. Pharm. Bull. 19 (7) 1301-1307 (1971)). Thus, these compounds are considered to act on the central nervous system and expected to be useful as a biologically active substance.

Conventionally, theanine is generally extracted from dried tea leaves obtained in tea plantations where refined green tea containing theanine is produced. However, this method has two defects, namely, (1) only about 1.5% theanine is stored per predetermined amount of dried tea leaves and (2) photosynthesis is actively carried out in ordinary tea plantations and accordingly, synthesized theanine is quickly resolved, whereupon an amount of stored theanine is small. Thus, it is difficult and not practical to produce a sufficient amount of theanine by the extraction from the dried tea leaves.

Accordingly, new industrial production methods of theanine have been proposed. As one of the methods, a chemical organic synthesis of theanine has been reported (Chem. Pharm. Bull. 19 (7) 1301-1307 (1971)). However, the organic synthesis reaction has a low yield and requires a complicated operation in separation and refinement of composition and the like. Furthermore, an enzyme method has been reported as another industrial production method. In this enzyme method, theanine is synthesized from L-glutamine and ethylamine through the use of γ-glutamyl radical group transition reaction of glutaminase derived from Pseudomonas (JP-A-H11-225789). Additionally, another enzyme method has been developed in which this enzyme is fixed to a carrier (JP-A-H05-328986). However, when glutaminase derived from Pseudomonas is used, L-glutamic acid is synthesized as side reaction product by hydrolysis reaction as well as theanine. Accordingly, L-glutamic acid as a by-product complicates refinement of theanine.

SUMMARY OF THE INVENTION

Therefore, an object of the present invention is to provide an efficient method of making theanine.

The inventors made research to overcome the above-described problem and found that theanine was able to be synthesized at high yield through the use of glutaminase derived from microbes of one or more of Bacillus, mold and yeast, and an amount of by-product was exceedingly small. Thus, the inventors completed the invention basically. More specifically, the present invention is directed to a method of making theanine wherein glutaminase derived from microbes of one or more of Bacillus, mold and yeast is caused to act on glutamine and ethylamine derivative.

The invention provides an efficient novel method of making theanine and can realize simple industrially advantageous production. More specifically, a higher conversion rate to theanine was admitted through the use of glutaminase derived from microbes of one or more of Bacillus, mold and yeast, whereupon industrial production is realized.

BRIEF DESCRIPTION OF THE DRAWINGS

Other objects, features and advantages of the present invention will become clear upon reviewing the following description of the embodiments with reference to the accompanying drawings, in which:

FIG. 1 shows an amount of synthesized theanine and an amount of glutamic acid in the case where enzymatic synthesis of theanine from L-glutamine and ethylamine is carried out through the use of glutaminase derived from Bacillus and glutaminase derived from Pseudomonas;

FIG. 2 shows an amount of synthesized theanine and an amount of glutamic acid in the case where enzymatic synthesis of theanine from L-glutamine and ethylamine is carried out through the use of glutaminase derived from mold and glutaminase derived from Pseudomonas;

FIG. 3 shows an amount of synthesized theanine and an amount of glutamic acid in the case where enzymatic synthesis of theanine from L-glutamine and ethylamine is carried out through the use of glutaminase derived from yeast and glutaminase derived from Pseudomonas; and

FIG. 4 is a graph showing IR spectrum of sample theanine and isolated substance.

DETAILED DESCRIPTION OF THE INVENTION

Embodiments of the present invention will be described in detail. However, the technical scope of the invention should not be limited by the following description of embodiments but can be practiced in various modified forms. Furthermore, it is noted that the technical scope of the invention should encompass the scope of equivalence.

Theanine used in the invention is a glutamic acid derivative contained in tea leaves and a principal component of deliciousness of tea. Theanine is used as a food additive for use as gustatory. More specifically, theanine is a compound such as γ-glutamilethylamide or L-glutamic acid-γ-ethylamide.

The ethylamine derivative used in the invention is ethylamine, ethylamine hydrochloride, ethylamine chloroaurate, ethylamine fatty acid salt, ethylamine picrate, N-benzenesulfonyl compound of ethylamine, N-p-toluenesulfonyl compound of ethylamine and the like. There is no specific limitation to them. Furthermore, ethylamine and ethylamine hydrochloride are particularly preferred.

Glutaminase used in the invention has glutaminase activity hydrolyzing L-glutamine thereby to produce L-glutamic acid and is used to improve taste of fermentative food such as Japanese "miso" and soy sauce. It is known that γ-glutamil transition activity is higher than hydrolytic activity in glutaminase under alkaline conditions. Glutaminase can also be used for synthesis of alkylamide such as theanine.

Glutaminase activity in the invention is measured by causing enzyme to react with L-glutamine serving as a substrate to determine L-glutamic acid produced. An amount of produced L-glutamic acid can be measured using a commercially available kit, for example, F kit L-glutamic acid (Roche Diagnostics). As the unit for the present enzyme, an amount of enzyme producing 1 □mol glutamic acid per min. is defined as "mU". Using this definition, an amount of enzyme per mg protein in a solution is defined as glutaminase activity mU/mg.

Bacillus used in the invention is cytomorphologically a bacteria having the characteristics of gram positive aerobic bacteria, bacillus, sporulatability, movability and the like.

There is no specific limitation to glutaminase derived from Bacillus in the invention. However, such glutaminase is preferably enzyme derived from Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus lentus, Bacillus licheniformis, Bacillus polymixa, Bacillus stearothermophilus or Bacillus thermoproteolyticus. From the viewpoint that bacteria with high glutaminase specific activity is preferred, glutaminase derived from Bacillus subtilis or Bacillus amyloliquefaciens is most preferred.

Glutaminase derived from Bacillus may be produced from modified bacteria such as gene recombination by application of biotechnology.

Mold in the invention is a general term for an indefinite aggregate with entangled mold hyphae in fungus and can be found in Phycomycetes, many of Ascomycetes and part of Basidiomycetes.

There is no specific limitation to glutaminase derived from mold in the invention. However, such glutaminase is preferably enzyme derived from Aspergillus oryzae, Aspergillus niger, Penicillium notatum, Rizopus stolonifer or Mucor sponosus. From the viewpoint that bacteria with high glutaminase specific activity is preferred, glutaminase derived from Aspergillus oryzae or Aspergillus niger is most preferred.

Furthermore, glutaminase derived from mold may be produced from modified bacteria such as gene recombination by application of biotechnology.

Yeast in the invention is a fungus belonging to Ascomycetes. The yeast contains no chlorophyll and breeds by gemmation and sometimes by division. The yeast is used for production of alcoholic beverages, soy sauce, bread and the like.

There is no specific limitation to glutaminase derived from yeast in the invention. However, such glutaminase is preferably enzyme derived from Saccharomyces cerevisiae, Saccharomyces rouxii, Candida utilis, Candida antarctica, Hansenulla anomala, Schizosaccaromyces octosporus. From the viewpoint that bacteria with high glutaminase specific activity is preferred, glutaminase derived from Saccharomyces cerevisiae, Saccharomyces rouxii, Candida utilis or Candida antarctica is most preferred.

Furthermore, glutaminase derived from yeast may be produced from modified bacteria such as gene recombination by application of biotechnology.

The aforesaid glutaminase derived from microbes of one or more of Bacillus, mold and yeast may be (1) microbes or (2) crude enzyme extracted from microbes. However, from the viewpoint of a theanine conversion ratio, it is preferable to refine glutaminase from microbes. Any conventional enzyme refining method may be employed for refinement of glutaminase. For example, column chromatography, partition with use of a solvent, dialysis, ultrafiltration, electrophoresis, fractional salting out by the use of normal salt, fractional precipitation by the use of alcohol or acetone, high performance liquid chromatography (HPLC) and the like can be exemplified. Of these, it is preferable to refine glutaminase by the combination of partition with use of a solvent, various types of chromatography and HPLC. Furthermore, glutaminase may be refined by combining one or more of glutaminase may be refined by the combination of CM-cellulose column chromatography, sephadex G150 column chromatography, hydroxyapatite column chromatography, butyl-Toyopearl® column chromatography.

Thus, in the method of making theanine according to the invention, glutaminase is derived from one or more of Bacillus, Penicillium, Rizopus, Mucor, Aspergillus, Hansenulla, Schizosaccaromyces and Candida. It is preferable to cause the glutaminase to act on glutamine and ethylamine derivative. In this case, (1) it is preferable that the microbes be cultured under the condition where culture supernatant of the microbes has a specific activity of not less than 10 mU/mg. Furthermore, (2) it is preferable that the glutaminase have a ratio (theanine/glutamic acid) of theanine as a main product to a glutamic acid as a by-product larger than 5, form the viewpoint of reducing by-product.

There is no specific limitation to fluidity in the synthesis of theanine enzyme in the invention. However, it is preferable that pH range from about 9 to 12. It is more preferable that pH range from about 10 to 11. Furthermore, there is no specific limitation to a reaction temperature. However, it is preferable that the reaction temperature range from about 0°C to 45°C. It is more preferable that the reaction temperature range from about 4°C to 30°C. There is no specific limitation to the densities of L-glutamine and ethylamine derivative. However, it is preferable that the density of L-glutamine be not less than about 0.1 mol and the density of ethylamine derivative be not less than about 1 mol.

L-glutamine in the invention includes pure L-glutamine and may contain suitable organic or inorganic salt, such as L-glutamine sodium salt.

Any known amino acid refining method may be used in order that theanine synthesized by the method of the invention may be isolated from reaction liquid and refined. For example, column chromatography, partition with use of a solvent, dialysis, crystallization, ultrafiltration, electrophoresis, fractional salting out by the use of normal salt, fractional precipitation by the use of alcohol or acetone, high performance liquid chromatography (HPLC) and the like can be exemplified. Of these, it is preferable to refine glutaminase by the combination of partition with use of a solvent, various types of chromatography and HPLC. Furthermore, glutaminase may be refined by combining one or more of glutaminase may be refined by the combination of

CM-cellulose column chromatography, sephadex G150 column chromatography, hydroxyapatite column chromatography, butyl-Toyopearl column chromatography.

The carrier in the invention fixes glutaminase. For example, the carrier may be an inorganic carrier such as Celite, silious earth, kaolinite, silica gel, molecular sieves, porous glass, activated charcoal, calcium carbonate, ceramics or the like or an organic high polymer such as ceramic powder, polyvinyl alcohol, polypropylene, chitosan, ion-exchange resin, chelate resin, synthetic adsorptive resin or the like. However, there is no specific limitation to the carrier in the invention.

The invention will be described in more detail by way of embodiments and test examples. These embodiments and test examples constitute a part of the embodiments of the invention but the invention should not be limited to the embodiments and test examples.

Embodiment 1:

Bacillus subtilis was cultured at 30°C in a culture medium of pH 7.0 containing 0.3% glucose, 3.0% polypeptone, 1.0% yeast extract and 0.5% sodium chloride. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA; Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 67 mU/mg.

Embodiment 2:

Bacillus amyloliquefaciens was cultured at 30°C in a culture medium of pH 7.0 containing 0.3% glucose, 3.0% polypeptone, 1.0% yeast extract and 0.5% sodium chloride. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 53 mU/mg.

Embodiment 3:

Bacillus coagulans was cultured at 30°C in a culture medium of pH 7.0 containing 0.3% glucose, 3.0% polypeptone, 1.0% yeast extract and 0.5% sodium chloride. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 43 mU/mg.

Embodiment 4:

Bacillus licheniformis was cultured at 30°C in a culture medium of pH 7.0 containing 0.3% glucose, 3.0% polypeptone, 1.0% yeast extract and 0.5% sodium chloride. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA; Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 40 mU/mg.

Embodiment 5:

Bacillus cereus was cultured at 30°C in a culture medium of pH 7.0 containing 0.3% glucose, 3.0% polypeptone, 1.0% yeast extract and 0.5% sodium chloride. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 5 mU/mg.

Compared example 1: Preparation of refined glutaminase derived from Pseudomonas nitroreducens

Pseudomonas nitroreducens was cultured in a 30 L jar fermenter (30 lit., ventilation 1 vvm=25 L/min., revolution 2000rpm) for about 20 hours using a culture fluid (pH 7) containing 0.6% sodium glutamate, 0.1% yeast extract, 1.0% glucose, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.07% $MgSO_4$□$7H_2O$ and 0.01% EDTA-Fe. After having been washed, bacterial cells in the obtained culture fluid were suspended in 7.5 L of 30 mM buffer solution of potassium phosphate (pH 7.0) and ultrasonically crushed in a temperature range from 5°C to 20°C, whereupon crushed bacterial cells were obtained.

The crushed bacterial cells were fractionated using ammonium sulfate while pH was adjusted to 7 by 7% ammonia water, whereby 45 to 90% saturation fraction was obtained. The obtained saturation fraction was dissolved into a buffer solution of 0.01 M potassium phosphate to be dialyzed. A dialysate was adsorbed using DEAE-cellulose column (15×60 cm) and glutaminase was eluted by a buffer solution containing 0.1 M salt, whereby glutaminase solution was obtained. The obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 15 mU/mg.

Example 6: Theanine enzyme synthesis by refined glutaminase

Using refined glutaminase (0.1 mL), theanine enzyme synthesis was carried out for 10 mL substrate solution (0.5 M L-glutamine and various densities of ethylamine) under the condition where pH was 10. 0 and the temperature was 30°C.

Embodiment 7: Determination of amount of theanine and glutamic acid

An enzyme reaction liquid in which theanine enzyme synthesis was executed was diluted suitably and thereafter, HPLC was carried out for the diluted reaction liquid so that amounts of theanine and glutamic acid were determined. A mol conversion ratio from an amount of glutamine (mol/L) of the substrate was calculated using obtained amounts of theanine and glutamic acid (mol/L). TABLE 1 shows the conditions for determination by HPLC.

TABLE 1

Analysis column:	Develosil ODS HG-5/Nomura Chemical Co., Ltd.
Detector:	Waters2487 Dual λ UV/VIS Detector/Waters
Sample of theanine:	L-theanine/Kurita Industry Co., Ltd.
Inner standard substance:	Nicotinoamide/NACALAI TESQUE, INC.

Mobile phase: Pure water:methanol:trifluoroacetic acid =980:20:1

Test example 1: Theanine enzyme synthesis by glutaminase derived from Bacillus and glutaminase derived from Pseudomonas

A theanine enzyme synthesis test was carried out under the conditions of embodiment 6 using glutaminase derived from each microorganism prepared in embodiments 1 to 5 and compared example 1. Amounts of theanine and glutamic acid after the test were measured under the conditions of embodiment 7. FIG. 1 shows the results of the test.

A mol conversion ratio from L-theanine to theanine is not less than 50% when glutaminase of each of embodiments 1 to 5 and compared example 1 is used. In particular, the mol conversion ratios of glutaminase of embodiments 1 to 4 reach high values of 78%, 76%, 72% and 70% respectively. On the other hand, regarding mol conversion ratios from L-glutamine to L-glutamic acid (production of by-product), glutaminase of each of embodiment 5 and compared example 1 has a high value of not less than 15% although glutaminase of each of embodiments 1 to 4 has a low value of not more than 6%. When theanine is synthesized using glutaminase, it is preferable that a mol conversion ratio to L-glutamic acid (by-product) be low as well as that a mol conversion ratio to theanine be high. As a result, the process of refining theanine can be simplified. In order that the aforesaid conditions may be met, it is preferable to use glutaminse derived from Bacillus and having a glutaminse specific activity of culture supernatant thereof not less than 10 mU/mg.

Embodiment 8:

Aspergilus oryzae was cultured at 30°C in a culture medium of pH 5.0 containing 2.0% malt extract, 2.0% glucose, 0.1% peptone and 0.1% yeast extract. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 42 mU/mg.

Embodiment 9:

Aspergilus niger was cultured at 30°C in a culture medium of pH 5.0 containing 2.0% malt extract, 2.0% glucose, 0.1% peptone and 0.1% yeast extract. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 39 mU/mg.

Embodiment 10:

Rizopus stolonifer was cultured at 30°C in a culture medium of pH 5.0 containing 2.0% malt extract, 2.0% glucose, 0.1% peptone and 0.1% yeast extract. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 15 mU/mg.

Embodiment 11:

Mucor sponosus was cultured at 30°C in a culture medium of pH 5.0 containing 2.0% malt extract, 2.0% glucose, 0.1% peptone and 0.1% yeast extract. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 5 mU/mg.

Test example 2: Theanine enzyme synthesis by glutaminase derived from mold and glutaminase derived from Pseudomonas

A theanine enzyme synthesis test was carried out under the conditions of embodiment 6 using glutaminase derived from each microorganism prepared in embodiments 8 to 11 and compared example 1. Amounts of theanine and glutamic acid after the test were measured under the conditions of embodiment 7. FIG. 2 shows the results of the test.

A mol conversion ratio from L-theanine to theanine is not less than 50% when glutaminase of each of embodiments 8 to 11 and compared example 1 is used. In particular, the mol conversion ratios of glutaminase of embodiments 8 and 9 reach high values of 72% and 73% respectively. On the other hand, regarding mol conversion ratios from L-glutamine to L-glutamic acid (production of by-product), glutaminase of compared example 1 has a high value of not less than 10% although glutaminase of each of embodiments 8 and 9 has a low value of not more than 5%. When theanine is synthesized using glutaminase, it is preferable that a mol conversion ratio to L-glutamic acid (by-product) be low as well as that a mol conversion ratio to theanine be high. As a result, the process of refining theanine can be simplified. In order that the aforesaid conditions may be met, it is preferable (1) that glutaminase is derived from mold (particularly, Asppergillus, Rozopus and Mucor) and has a specific activity of not less than 10 mU/mg or (2) that the glutaminase has a ratio (a ratio of theanine/glutamic acid=X) of theanine as a main product to a glutamic acid as a by-product represented as X>5 in a mol conversion ratio from L-glutamine.

Embodiment 12:

Saccharomyces cerevisiae was cultured at 30°C in a culture medium of pH 5.0 containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 45 mU/mg.

Embodiment 13:

Saccharomyces rouxii was cultured at 30°C in a culture medium of pH 5.0 containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 40 mU/mg.

Embodiment 14:

Candida utilis was cultured at 30°C in a culture medium of pH 5.0 containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA; Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 30 mU/mg.

Embodiment 15:

Candida antarctica was cultured at 30°C in a culture medium of pH 5.0 containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 25 mU/mg.

Embodiment 16:

Hansenulla anomala was cultured at 30°C in a culture medium of pH 5.0 containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1.0% glucose. An obtained culture fluid was processed by a centrifugal separator, whereupon culture supernatant was obtained. Cold ethanol was added to the culture supernatant, and obtained precipitation was processed by a centrifugal separator and then recovered. Obtained precipitate was dissolved into a buffer solution of phosphoric acid (pH 7.0) and then dialyzed. A dialysate was adsorbed using DEAE-Sepharose Fast Flow and thereafter, the purity of protein was improved by elution by salt solution. Obtained glutaminase solution was condensed and desalinated using a UF film (UFP-5-C-3MA, Amersham Bioscience KK), whereby refined glutaminase was obtained. Glutaminase specific activity of the culture supernatant was 15 mU/mg.

Test example 3: Theanine enzyme synthesis by glutaminase derived from yeast and glutaminase derived from Pseudomonas

A theanine enzyme synthesis test was carried out under the conditions of embodiment 6 using glutaminase derived from each microorganism prepared in embodiments 12 to 16 and compared example 1. Amounts of theanine and glutamic acid after the test were measured under the conditions of embodiment 7. FIG. 3 shows the results of the test.

A mol conversion ratio from L-theanine to theanine is not less than 50% when glutaminase of each of embodiments 12 to 16 and compared example 1 is used. In particular, the mol conversion ratios of glutaminase of embodiments 12 to 15 reach high values of not less than 70% respectively. On the other hand, regarding mol conversion ratios from L-glutamine to L-glutamic acid (production of by-product), glutaminase of compared example 1 has a high value of not less than 10% although glutaminase of each of embodiments 12 to 15 has a low value. When theanine is synthesized using glutaminase, it is preferable that a mol conversion ratio to L-glutamic acid (by-product) is low as well as that a mol conversion ratio to theanine be high. As a result, the process of refining theanine can be simplified. In order that the aforesaid conditions may be met, it is preferable that glutaminase is derived from yeast (particularly, Saccharomyces and Candida) and has a specific activity of not less than 10 mU/mg.

Embodiment 17: Preparation of fixed glutaminase using CHITOPEARL® 4010

CHITOPEARL® 4010 (manufactured by Fuji Spinning Co., Ltd.) was immersed in a buffer solution of 50 mM sodium phosphate (pH 7.4) for 24 hours. After equilibration, 10 mL CHITOPEARL® 4010 was immersed in 25 mL glutaminase (15 mg/mL) prepared in embodiment 3 was shaken for about two hours. Thereafter, CHITOPEARL® 4010 from which adherent liquid had been removed was added to 2.5% glutaraldehyde and then shaken for two hours. After process of glutaraldehyde, CHITOPEARL® 4010 was washed using a buffer solution of 50 mM sodium phosphate (pH 7.4) an amount of which is 30 times larger than an amount of CHITOPEARL® 4010. The washing was continued until absorbance (280 nm) became equal to or smaller than 0.01. CHITOPEARL® 4010 was charged into a column.

Embodiment 18: Enzyme reaction by fixed glutaminase

With use of the fixed glutaminase prepared in embodiment 17, a substrate solution (4% glutamine and 25% ethylamine, pH 10.0) was passed through the column at a flow rate SV=0.2 at 30°C. As a result, theanine was able to be obtained at the yield of 70%.

Embodiment 17: Preparation of fixed glutaminase using anion exchange resin

10 mL DIAION® HPA25 (Mitsubishi Chemical Corporation) which is an anion exchange resin was added to 25 mL refined glutaminase (15 mg/mL) obtained in embodiment 3. Subsequently,

the mixture was shaken for about two hours. Thereafter, DIAION® HPA25 from which adherent liquid had been removed was added to 2.5% glutaraldehyde solution and then shaken for further two hours. After process of glutaraldehyde, DIAION® HPA25 was washed using a buffer solution of 50 mM sodium phosphate (pH 7.4) an amount of which is 30 times larger than an amount of CHITOPEARL® 4010. The washing was continued until absorbance (280 nm) became equal to or smaller than 0.01. DIAION® HPA25 was charged into a column.

Embodiment 20: Enzyme reaction by fixed glutaminase

With use of the fixed glutaminase prepared in embodiment 19, a substrate solution (4% glutamine and 25% ethylamine, pH 10.0) was passed through the column at a flow rate SV=0.2 at 30°C. As a result, theanine was able to be obtained at the yield of 70%.

Embodiment: Refinement of theanine

In isolation and refinement of theanine from reaction liquid, ethylamine was removed from the reaction liquid by vacuum concentration and thereafter, desalination was carried out using a reverse osmosis (RO) membrane. Subsequently, the reaction liquid was applied to column chromatography of Dowex 50×8 and Dowex 1×2 and then treated by ethanol.

When applied to an amino acid analyzer and a paper chromatography, the isolated substance exhibited the same behavior as a standard substance of theanine. Furthermore, hydrolysis of the isolated substance using hydrochloric acid or glutaminase yielded L-glutamine and ethylamine at a ratio of 1:1. Thus, since hydrolysis of the isolated substance by glutaminase was thus possible, ethylamine was proved to be combined with the gamma-position of L-glutamine. Furthermore, it was confirmed by L-glutamic acid dehydrogenaze (GluDH) that glutamine yielded by hydrolysis was of L-type. FIG. 4 shows infrared absorption spectrometry (IR) spectra of theanine sample and isolated substance. Both substances exhibited spectra similar to each other. Consequently, the isolated substance was proved to be theanine.

The foregoing description and drawings are merely illustrative of the principles of the present invention and are not to be construed in a limiting sense. Various changes and modifications will become apparent to those of ordinary skill in the art. All such changes and modifications are seen to fall within the scope of the invention as defined by the appended claims.

2005257281 22 Jul 2010

CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of making theanine comprising the steps of:

deriving a glutaminase from microbes of one or more of Bacillus amyloliquefaciens, Bacillus coagulans, Aspergilus niger, Rizopus stolonifer, Saccharomyces cerevisiae, Saccharomyces rouxii, Candida utilis and Candida antarctica ; and

causing the glutaminase to act on glutamine and an ethylamine derivative to synthesize the theanine, wherein (i) the microbes are cultured so that a culture supernatant of the microbes has a specific activity of not less than 10 mU/mg, (ii) a ratio of the theanine derived from the glutaminase as a main product to glutamic acid derived from the glutaminase as a by-product is larger than 5, and (iii) the method has a pH in a range of 9 to 12,

the mol conversion ratio from L-glutamine to theanine is more than 70%, and

the mol conversion ratio from L-glutamine to glutamic acid is not more than 6%.

2. The method according to claim 1, wherein the glutaminase is fixed to a carrier.

FIGURE 1

TYPES OF MICROBES		EMBODIMENT1 Bacillus subtilis	EMBODIMENT2 Bacillus amyloliquefaciens	EMBODIMENT3 Bacillus coagulans	EMBODIMENT4 Bacillus licheniformis	EMBODIMENT5 Bacillus cereus	COMPARED EXAMPLE1 Pseudomonas nitroreducens
GLUTAMINASE SPECIFIC ACTIVITY OF CULTURE SUPERNATANT (mU/mg)		67	53	43	40	5	15
MOL CONVERSION RATIO FROM L-GLUTAMINE (%)	THEANINE	75	74	70	69	55	50
	GLUTAMIC ACID	2	3	5	7	10	10

FIGURE 2

TYPES OF MICROBES		EMBODIMENT8 Aspergillus oryzae	EMBODIMENT9 Aspergillus niger	EMBODIMENT10 Rizopus stolonifer	EMBODIMENT11 Mucor sponosus	COMPARED EXAMPLE1 Pseudomonas nitroreducens
GLUTAMINASE SPECIFIC ACTIVITY OF CULTURE SUPERNATANT (mU/mg)		42	39	15	5	15
MOL CONVERSION RATIO FROM L-GLUTAMINE (%)	THEANINE	72	73	65	52	50
	GLUTAMIC ACID	5	4	5	9	10

FIGURE 3

TYPES OF MICROBES		EMBODIMENT12 Saccharomyces cerevisiae	EMBODIMENT13 Saccharomyces rouxii	EMBODIMENT14 Candida utilis	EMBODIMENT15 Candida antarctica	EMBODIMENT16 Honsenulla anomala	COMPARED EXAMPLE1 Pseudomonas nitroreducens
GLUTAMINASE SPECIFIC ACTIVITY OF CULTURE SUPERNATANT (mU/mg)		45	40	30	25	15	15
MOL CONVERSION RATIO FROM L-GLUTAMINE (%)	THEANINE	75	70	71	72	54	50
	GLUTAMIC ACID	4	5	6	5	11	10

FIGURE 4