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1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
*tel.* 202.434.4100
*fax* 202.434.4646

**Writer's Direct Access**
**John B. Dubeck**
(202) 434-4125
Dubeck@khlaw.com

November 10, 2008

**Via Federal Express**

Dr. Laura M. Tarantino
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: Submission of GRAS Notification for Protein-Glutaminase Enzyme Derived from *Chryseobacterium proteolyticum*

Dear Dr. Tarantino:

Pursuant to proposed 21 C.F.R. § 170.36(c) and on behalf of our client, Amano Enzyme, Inc., (Amano) of Nagoya, Japan, we hereby notify the agency of our determination on the basis of scientific procedures that Amano's Protein-Glutaminase enzyme preparation is generally recognized as safe (GRAS) when used to enhance the functionality of protein as described in the enclosed notification. As with all GRAS substances, the described use of Protein-Glutaminase is exempt from the premarket clearance requirement applicable to food additives under section 409 of the Food, Drug, and Cosmetic Act.

We trust you will find the enclosed notification acceptable. Should any questions arise during the review process, please do not hesitate to contact us, preferably by telephone, so that we may respond as quickly as possible.

Sincerely,

000002

John B. Dubeck

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OFFICE OF
FOOD ADDITIVE SAFETY

cc: Mr. Atsushi Mizutani

Enclosure: GRAS Notification in triplicate

# GRAS Notification for Protein-Glutaminase derived from *Chryseobacterium proteolyticum*

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## 1. Claim Regarding GRAS Status

Amano Enzyme Inc. hereby notifies the Food and Drug Administration ("FDA") through its attorneys that its Protein-Glutaminase enzyme preparation (hereinafter "PG" or "PG Enzyme"), derived from *Chryseobacterium proteolyticum*, is GRAS when used to enhance the functionality of protein, as described in greater detail below. As such, PG Enzyme is exempt from the premarket approval requirements of the Food, Drug, and Cosmetic Act.

i. Name and Address of the Notifier

Mr. Shinya Hayashi
Amano Enzyme Inc.
2-7, 1-Chome, Nishiki
Naka-ku, Nagoya 460-8630
JAPAN

ii. Common or Usual Name of the Subject Substance

The proposed common or usual name of the notified substance is:

Protein-Glutaminase derived from *Chryseobacterium proteolyticum (C. proteolyticum)*

iii. Conditions of Use

PG Enzyme is useful in the processing of various types of protein such as casein, whey protein, dried egg white, soy, and wheat protein. When protein ingredients are treated with the enzyme, it is typically applied at a level of 0.49% by weight of the protein ingredient. The PG Enzyme may also be added directly to a wide variety of processed foods such as breads, noodles, tofu, fish- and meat-derived foods, cheese, certain beverages, and seasonings. When added to food, the enzyme is typically used at a rate of 0.09% of the protein content of the food. By either use pattern, the modified proteins are useful in a wide variety of foods for their ability to impart desirable changes to texture, flavor, or physical functionality.

The manner of the PG Enzyme use is similar to that of proteases; however, unlike amylases used in the production of corn syrup, PG Enzyme protein will remain in the treated proteins. PG Enzyme activity, however, is inactivated by heat treatment (such as 70℃ for 10 min). PG Enzyme functions by deamidating protein. When a protein has been processed with 0.49% PG Enzyme, virtually complete deamidation is achieved. As a result desireable impacts on texture, flavor or physical functionality can be obtained by using relatively small amounts of the treated protein when formulating finished foods.

PG catalyzes the hydrolysis of protein-bound glutaminyl residues to release ammonia without cleaving peptide bonds or producing other undesirable changes in protein structure. In other words, the enzyme converts glutaminyl residues in protein to glutamyl residues. This improves protein functionality in food. It allows the modified protein to be used in small amounts for emulsification, foaming, gelling, or other functional uses.

The most striking improvement attributed to the use of PG Enzyme is an increase of solubility, because the formed negative charge of glutamic acid would contribute the electrostatic repulsion of intermolecular proteins. Insoluble wheat gluten can be solubilized without any peptide bond cleavage.

Other possible applications of PG Enzyme are dough softening for baking (US2004072318, Example 16, 17), increase of protein solubility in the presence of calcium (US2004072318, Example 19), enzymatic protein hydrolyzate (US2004072318, Example 20), and reduction of food protein allegenicity (J. Agric. Food Chem., 54:6034, 2006). PG can be also used for the control of transglutaminase reaction that is used for the alteration of food texture through protein cross-liking (US2004072318, Example 23).

When added directly to processed foods, the effect of the PG Enzyme on protein is the same as for protein ingredient treatment; protein components are deamidated. Using too much PG may have adverse effects texture, flavor or physical properties. For example, when used for dough softening, using too much PG yields a sticky dough that is very difficult to handle. Thus, a lower rate of use is recommended for direct addition to processed foods.

iv. Basis for the GRAS Determination

Amano's GRAS determination for PG Enzyme is based upon scientific procedures, as described in greater detail in Section 4 below.

v. Statement of Availability of Data and Information

The data and information that are the basis for Amano's GRAS determination are available for review and copying by FDA at the offices of Keller and Heckman, LLP, 1001 G Street, N.W., Suite 500 West, Washington, D.C. 20001. These documents will be sent to FDA upon request.

Robert J. Scheuplein, Ph.D. Date
Staff Scientist
Keller and Heckman, LLP
1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
202-434-4100

John B. Dubeck Date
Attorney for the Notifier
Keller and Heckman, LLP
1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
202-434-4100

## 2. Detailed Information About the Identity of the Notified Substance

i. Name

Protein glutaminase

ii. Enzyme Commission Number

3.5.1.XX

iii. Production Organism

*Chryseobacterium proteolyticum*

iv. Physical Description

White to yellowish white powder.

v. Method of Manufacture

PG Enzyme is produced by the controlled fermentation of a selected strain of *C. proteolyticum*, isolated from soil. PG catalyzes the hydrolysis of protein-bound glutaminyl residues to release ammonia without cleaving peptide bonds or producing other undesirable changes in protein structure. The strain has not been subjected to recombinant DNA technology. The fermentation medium is prepared from food-grade ingredients.

The PG Enzyme activity is separated from the production organism following fermentation by a series of filtration steps and ion-exchange chromatography. The production organism is removed through microfiltration. The product is mixed with a bulking agent, spray dried, sieved, and mixed. The finished enzyme preparation conforms to the purity criteria for enzyme preparations established in the *Food Chemicals Codex* (6th Ed.), including the general and additional requirements. The manufacturing process is presented diagramatically in Appendix 1.

Amano has published an article describing the development of the enzyme in more detail.[1]

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[1] Yamaguchi & Yokoe, A Novel Protein-Deamidating Enzyme from *Chyrseobacterium proteolyticum* sp. nov., a Newly Isolated Bacterium from Soil. *Appl. Environ. Microbiol.* Vol. 66, No. 8 (Aug. 2000), p 3337-3343.

vi. Specifications

PG Enzyme preparation satisfies the purity criteria for Enzyme Preparations found in the 5th Edition of the *Food Chemicals Codex* including the General Requirements and the Additional Requirements described on pages 151-152.

## 3. Information Relevant to Self-limiting Levels of Use

Not applicable.

## 4. Detailed Summary of Basis for Notifier's GRAS Determination

Amano's GRAS determination is based upon a series of toxicological studies of the enzyme and of the production organism. These studies include a 90-day gavage study with rats, genotoxicity studies, and a pathogenicity and toxigenicity study of the production organism using mice. A published article describing these studies in detail is presented as Appendix 2.

i. 90-Day Rat Gavage Study

On the basis of a preliminary two-week oral study, in which no treatment related effects were observed at the second highest dose level, 1269mg/kg body wt/day, groups of 12 male and 12 female Sprague-Dawley SPF rats [Crj:CD(SD)IGS], 6 weeks of age at the initiation of dosing, received PG Enzyme (activity 549 U/g) by gavage at levels of 0, 635, 1269, and 2538 mg/kg body wt/day. Animals were observed three times daily (twice on Saturdays and holidays) for clinical signs. Body weight was measured three times during week one of the study and twice weekly thereafter.

No deaths occurred during the administration or recovery periods, and there were no clinical signs or ophthalmological abnormalities. Mean food consumption and body weights did not differ significantly from the controls. There were no treatment-related changes in hematology parameters relative to the controls. A more complete discussion of the methods and observations recorded in this study is provided in the article presented as Appendix 2. The no-observed-adverse-effect-level (NOAEL) was set at 2538 mg/kg body weight, the highest dose level tested. Application of a 100-fold safety factor yields an acceptable daily intake (ADI) of 25 mg/kg body weight for humans.

ii. Genotoxicity Studies

a. Reverse Mutation Test on Bacteria

Mutagenicity of PG Enzyme was evaluated in three strains of *Salmonella typhimurium* (TA100, TA1535, and TA1537) and in *E. coli* WP2*uvrA*. The dose-ranging was carried out with doses of 50, 150, 500, and 5000 μg/plate, the OECD recommended maximum test concentration for noncytotoxic substances. In a preliminary test, no growth inhibition was observed by PG Enzyme (activity 549 U/g) on any of the tester strains, with or without metabolic activation, at the highest level tested, 5000 μg/plate. Therefore, in the final tests, 5000 μg/plate was selected as the maximum test dose, with lower doses decreasing in half multiples (0, 313, 625, 1250, and 2500 μg/plate) for each strain with and without metabolic activation.

PG Enzyme did not increase the number of revertant colonies at doses from 313 to 5000 μg/plate with or without metabolic activation.

b. Chromosome Aberration Test

The potential of PG Enzyme to induce chromosome aberrations was tested using CHL/IU cells, derived from fibroblasts from the lungs of Chinese hamsters. There was no statistically significant increase in cells with structural chromosome aberrations or in polyploidy cells at any dose of PG Enzyme. A published article describing this study in further detail is presented as Appendix 2.

iii. Pathogenicity Study

Although the production organism is not present in the PG Enzyme preparation, it is preferable not to use pathogenic organisms for the production of enzymes intended for use in food applications.

*C. proteolyticum* was tested for pathogenicity and toxigenicity by intravenous injection and oral inoculation in mice.

For the tail vein injection study, the following were tested: two different concentrations of a cell suspension of *C. proteolyticum* ($2.9 \times 10^7$ and $2.9 \times 10^8$ cfu/mouse), a supernatant of culture broth, and a supernatant of cell lysate. Additionally, a saline solution was used as a control, and *P. aeruginosa* cell suspension was used as a positive control. Animals were observed for mortality and clinical signs over a 14-day period. Organs were removed for histopathological examination and examination for viable cells.

No deaths were noted throughout the observation period in the saline controls or in any *C. proteolyticum* test group. No viable cells were detected in any of the organs examined. Focal necrosis in the liver was observed in four of ten animals in the high-dose, *C. proteolyticum* cell suspension group ($2.9 \times 10^8$ cfu/mouse) and in one of ten animals in the supernatant lysate group. By contrast, six of the *P. aeruginosa*-dosed animals died, with viable cells and organ damage detected in the *P. aeruginosa*-dosed animals.

In the oral inoculation study, a *C. proteolyticum* cell suspension was administered at a dose of 1.25 x $10^9$ cfu/mouse. No deaths or abnormal symptoms were observed; no viable cells were detected in any of the organs examined; no histological findings were observed except focal necrosis in the liver of one of the five animals dosed.

These studies are described in more detail in the attached article. The *C. proteolyticum* production organisms was determined to be nonpathogenic and nontoxigenic on the basis of these results.

iv. Toxin and Antibiotic Analysis

a. Toxin

It was demonstrated that the PG-producing strain produced no toxin by the experiments described in section *3.1. Pathogenicity and toxigenicity study for the producing strain* in the paper of *Regul Toxicol Pharmacol.* 2007 Nov; 49(2):79-89. The studies include the intravenous and oral inoculation of both culture supernatant and cell extract, and also an endotoxin determination in the culture supernatant.

The literature search was performed on the following two kinds of data sources:

- The books recommended by Pariza and Johnson, *Bacterial Protein Toxins* (K.Aktories & I.Just eds, Springer-Verlag, 2000), *Guidebook to Protein toxins and their use in cell biology* (R.Rappuoli & C.Montecucco,Oxford Univ. press,1997), *The Comprehensive Sourcebook of Bacterial Protein Toxins*, second edition (J.E.Alouf & J.H.Freer eds, Academic press, 1999).
- PubMed database provided by NCBI.

No literature was found indicating that toxins are produced by the PG-producing strain or related strains.

b. Antibiotic

Examination of antibiotic activities of PG preparation has been carried out by the Quality Control Dept., Amano Enzyme Inc. (Nagoya, Japan) according to the FAO/WHO JECFA protocol for specifications for identity and purity of certain food additives. PG preparation had no antibiotic activity. The results are detailed in the Analytical Report dated May 9, 2008.

v. Safety Determination

To summarize, the NOAEL in the 90-day toxicity study in rats was set at 2538 mg/kg body weight per day for the PG Enzyme, yielding and ADI of 25 mg/kg bw for humans. PG Enzyme was found to be non-mutagenic, and the production organism was found to be nonpathogenic and nontoxigenic.

The estimated daily intake for PG Enzyme resulting from the application covered by this notification is no more than (and probably significantly less than) 580 mg/person/day. This compares favorably with the 25 mg/kg bw ADI, which corresponds to 1500 mg/person/day for a 60-kg adult.

vi. Intake Estimate

If we consider the total amount of protein present in the American diet and assume very conservatively that all of it might be treated with the PG Enzyme, we can demonstrate that the NOAEL from the 90-day rat study is high enough to support the safety of the intended use. This approach exaggerates actual dietary exposure to the enzyme because we expect only a small fraction of protein in the diet to be treated with the enzyme, in cases where the functionality of the treated protein is desirable. Nevertheless, under this approach, the resulting EDI is comfortably below the ADI. For this reason, we consider a more detailed EDI analysis unnecessary.

According to food consumption surveys, protein makes up less than 20% of the caloric value of the American diet.[2] In the case of a 2000 calorie diet, this translates to less than 100 grams of protein per person per day (2000 cal x 20% ÷ 4 cal/gram of protein). By comparison, FDA's "daily reference value" for protein in a 2000-calorie diet is 50 grams.[3]

FDA has produced a guidance document entitled "Guidance for Industry, Estimating Dietary Intake of Substances in Food." Under FDA's approach, an EDI is generally developed for heavy consumers of the food or group of foods in question. Following this approach, a very conservative EDI for the PG Enzyme can be calculated if we assume that a heavy consumer will ingest approximately 100 grams of protein per day occurring naturally in foods, with the possibility of an additional 100 grams contained in protein-enriched foods or supplements and in foods that contain protein added for a functional effect.

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2 *See*, for example, "Trends in Intake of Energy and Macronutrients – Untied States, 1971—2000," Morbidity and Mortality Weekly Report, February 6, 2004 / 53(04);80-82.

3 *See* 21 C.F.R. 101.9(c)(9). This value is used in FDA's nutrition labeling regulations and represents the amount of protein FDA expects to be present in a 2000-calorie diet.

If all protein present in the processed foods is assumed to be treated with PG Enzyme, at the typical rate of 0.09% of the protein content of the food, this corresponds to 90 mg of PG Enzyme per day (100 g protein x 0.09%). If all protein ingredients in the protein-enriched foods are assumed to be treated with PG Enzyme at the typical rate of 0.49%, this corresponds to 490 mg of PG Enzyme per day (100 g protein x 0.49%). The combined total PG intake under this approach is 580 mg/person/day. Again, we emphasize that only a small fraction of protein in the diet will actually be treated with the PG Enzyme. Furthermore, very few consumers are expected to consume 200 grams of protein per day. But, our approach demonstrates that reasonably foreseeable daily intake of the enzyme clearly will not exceed a safe level.

vii. Evaluation of PG Enzyme under the Pariza and Johnson Decision Tree

In a well-received article published in *Regulatory Toxicology and Pharmacology*, Michael Pariza and Eric Johnson present guidelines for evaluating the safety of production strain metabolites that are present in an enzyme preparation.[4] The authors consider the safety of the production strain the primary issue in evaluating enzyme safety, specifically the toxigenic potential of the production strain.

The decision tree outlines twelve steps for determining the safety of the production strain, only some of which apply if the strain is not genetically modified using rDNA techniques.[5] Specifically, for non-genetically modified strains that have not previously been evaluated by this method, the questions focus on whether the strain is

(1) nonpathogenic
(2) free of antibiotics
(3) free of oral toxins (or below levels of concern) known to be produced by other members of the same species, and
(4) shown to have a NOEL sufficiently high to ensure safety.

Because it is not genetically modified using rDNA techniques, the safety evaluation of PG Enzyme under the decision tree involves only the four questions set forth above. In particular, the production organism used to manufacture PG Enzyme is nonpathogenic, free of antibiotics, and free of oral toxins. As discussed above, the NOAEL for PG Enzyme was set at 2538 mg/kg body weight per day in a 90-day study in rats, the highest dose level tested. This easily accommodates dietary exposure to the enzyme associated with the intended use.

Thus, the PG Enzyme production organism, *C. proteolyticum*, can be deemed safe under the approach suggested by Pariza and Johnson, which is reflective of current scientific

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4 *See* Pariza, Michael and Eric Johnson, "Evaluating the Safety of Microbial Enzyme Preparations Use in Food Processing: Update for a New Century," Regulatory Toxicology and Pharmacology, 33: 173-186, (2001).

5 *Id.*

thinking regarding the evaluation of microbial enzyme preparations used in food processing.

viii. Expert Consensus

The safety studies described above for PG Enzyme and the production organism have been published in a peer-reviewed scientific journal,[6] a copy of which is presented as Appendix 2. The authors concluded that PG Enzyme may be regarded as safe when used as a processing aid in the food industry. It is unlikely that an expert would disagree with this conclusion, given the lack of toxicity associated with enzymes, the low estimated dietary exposure associated with this application, the safety of the production organism, and the margin of safety between the 90-day NOAEL for this particular enzyme and its conservatively estimated daily intake described above.

Thus, the expert consensus and the general recognition elements of GRAS status are satisfied by the fact that our published and peer reviewed safety evaluation is consistent with current scientific thinking regarding the evaluation of microbial enzyme preparations used in food processing.

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6 Scheuplein, Robert J., Atsushi Mizutani and Shotaro Yamaguchi, Studies on the non-pathogenicity of *Chryseobacterium Proteolyticum* and on the safety of the enzyme: Protein-glutaminase, Regulatory Toxicology and Pharmacology, 49: 79-89 (2007). *See* Appendix 2.

## List of Appendices


Appendix 1: Manufacturing Method for PG Enzyme

Appendix 2: Scheuplein, Robert J., Atsushi Mizutani and Shotaro Yamaguchi, Studies on the non-pathogenicity of *Chryseobacterium Proteolyticum* and on the safety of the enzyme: Protein-glutaminase, Regulatory Toxicology and Pharmacology, 49: 79-89 (2007).

1

# Composition and Specification of Protein-glutaminase Enzyme Preraration

There are 2 type of protein-glutaminase (PG) enzyme preparations which distinguished by deamidating activity (50 U/g and 500 U/g of activity).

Typical composition of PG500 (500 U/g) enzyme preparation is indicated in the table 1. Another preparation, PG50 is produced by 10 times dilution of PG500 using bulking agent.

Specification of PG500 enzyme preparation and the result of specification analysis are indicated in the table 2 and table 3, respectively. Specification of PG-50 is the same as PG-500 except enzyme activity. The purity of PG is estimated as more than 95% except bulking agent, as judged by SDS-polyacrylamide gel electrophoresis.

Table 1 Composition of PG enzyme preparation

| Name | Activity | Composition (%) | |
|---|---|---|---|
| | | Active constituent | Bulking agent* |
| PG-500 | More than 500u/g | 10.0 | 90.0 |

*: Dextrin. Including dextrin added in a spray-drying process.

Table 2 Specification of PG500 enzyme preparation

| Item | Method | Limiting Value |
|---|---|---|
| Activity<br>(Deamidating activity) | *see Appendix* | More than 500 U/g |
| Loss on drying | 1g, 105°C, 4hrs | Less than 10 % |
| Lead | Atomic absorption method | Less than 5ppm |

| | | |
|---|---|---|
| Total aerobic count | SCD agar plate method | Less than 1000 /g |
| Coliforms | FDA-BAM* method | Less than 30 /g |
| *E.. coli* | FDA-BAM method | Negative in 25 g |
| *Salmonella* | FDA-BAM method | Negative in 25 g |

* : U.S. Food and Drug Administration-Bacteriological Analytical Manual

Table 3 Results of specification analysis of PG500 enzyme preparation

| Item | PGE0952601K | PGF0451601K | PGG0352501K |
|---|---|---|---|
| Activity (Deamidating activity) | 565U/g | 520U/g | 537U/g |
| Loss on drying | 5.62% | 5.71% | 5.11% |
| Lead | Less than 1ppm | Less than 1ppm | Less than 1ppm |
| Total aerobic count | Less than 10 /g | Less than 10 /g | 1.0×10 /g |
| Coliforms | Less than 3.6/g | Less than 3.6/g | Less than 3.6/g |
| *E.. coli* | Negative/25g | Negative/25g | Negative/25g |

| | | | |
|---|---|---|---|
| *Salmonella* | Negative/25g | Negative/25g | Negative/25g |

Appendix

## Assay method for Deamidating activity

## 1 Reagents and Solutions

1) 0.02mol/L phosphate buffer (pH 7.0)

Dissolve 2.722g of potassium phosphate with water and adjust the volume to 1000mL (solution I). Dissolve 2.839g of anhydrous disodium phosphate with water and adjust the volume to 1000mL (solution II). Add an appropriate volume of solution I to solution II and adjust the pH to 7.0.

2) 0.176mol/L phosphate buffer (pH 6.5)

Dissolve 23.95g of potassium phosphate with water and adjust the volume to 1000mL (solution III). Dissolve 24.98g of anhydrous disodium phosphate with water and adjust the volume to 1000mL (solution IV). Add an appropriate volume of solution III to solution IV and adjust the pH to 6.5.

3) trichloroacetic acid solution

Dissolve 65.36g of trichloroacetic acid with water and adjust the volume to 1000mL.

4) coloring reagent A

Dissolve 40.46g of phenol and 0.15g of Sodium pentacyanonitrosylferrate(III) dihydrate with water and adjust the volume to 1000mL. Keep cold (4℃) and under light shielding.

5) coloring reagent B

Dissolve 49.94g of potassium hydrate with water and adjust the volume to 1000mL. Keep cold (4℃).

6) coloring reagent C

Dissolve 200.40g of anhydrous potassium carbonate and 8.33mL of sodium hypochlorite (food additive grade) with water and adjust the volume to 1000mL. Prepare a fresh solution daily.

7) Standard ammonia solution

Dissolve 0.314g of ammonium chloride with water and adjust the volume to 1000mL. Dilute 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0mL of ammonia solution in water and adjust the each volume to 10mL (final ammonia concentration are 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μg/mL, respectively). After dilute 0.06mL of each ammonia solution with 0.24mL of water, add 0.3mL of coloring reagent A and mix. Then, add 0.15mL of coloring reagent B and mix. Finally, add 0.3mL of coloring reagent C and mix. Incubate the mixture at 37±0.5℃ for

20minite and cool the final mixture in flowing water. Measure the absorptions at 630nm against the water blank in each solution. Prepare a standard curve by plotting the absorbances against the ammonia concentration and calculate the slope value (a) of the linear curve (Y=aX). Prepare a fresh solution daily.

8) Substrate solution (10mM Benzyloxycarbonyl-L-glutaminy-glycine solution)

Dissolve 0.337g of Benzyloxycarbonyl-L-glutaminylglycine (Cbz-Gln-Gly) with 0.176mol/L phosphate buffer (pH6.5) and adjust the volume to 100mL. Prepare a fresh solution daily.

9) Test sample solution

Dissolve an appropriate amount of test sample with diluent (0.02 mol/L phosphate buffer (pH7.0) or water or other applicable buffer or saline solution). Test sample solution should be prepared between 0.04 and 0.15u/mlL, within the linear range of the increase of generated ammonia concentration when carried out this assay. Prepare a fresh solution daily.

## 2 PROCEDURE

Pipet 0.1mL of test sample solution and incubate at 37±0.5℃ for 1 minute, add 1.0mL of substrate solution which preliminarily incubated at same temperature for 10minutes and mix. After Incubate at 37±0.5℃ for 60 minutes, add 1mL of trichloroacetic acid solution and mix to stop the reaction. Add 0.06mL of the mixture to 0.24mL of water and mix. Add 0.3mL of coloring reagent A and mix. Add 0.15mL of coloring reagent B and mix. Add 0.3mL of coloring reagent C and mix. Incubate the final mixture at 37±0.5℃ for 20minutes and cool in flowing water. Measure the absorption at 630nm against the water blank (A1).

For the reagent blank, pipet 0.1mL of test sample solution, add 1.0mL of trichloroacetic acid solution and incubate at 37±0.5℃ for 60minutes. After that, perform the same procedure described above and measure the absorption at 630nm (A2).

## 3 CALCULATIONS

One unit is defined as the quantity of enzyme that will produce 1 $\mu$ mol of ammonia per 1minute under this conditions.

Activity (u/g) $=(A1-A2) \times 2.1/0.1 \times 1/17.03 \times 1/60 \times 1/a \times 1000/W$

A1 : Absorbance of the sample reaction solution

A2 : Absorbance of the reagent blank solution

a : Slope value on the standard ammonia curve

W : Weight of the test sample in the test sample solution

2.1 / 0.1 : voluminal ratio of the reaction solution to the test sample solution

1/17.03 : conversion factor for mole value

1 /10 : conversion factor for per minute

2

Pages 000022 - 000032 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

SUBMISSION END

# *Reference List for Industry Submission, GRN 000267*

| *Pages* | *Author* | *Title* | *Publish Date* | *Publisher* | *BIB_Info* |
|---|---|---|---|---|---|
| 000022 - 000032 | Scheuplein, Robert J.; Mizutani, Atsushi; Yamaguchi,Shotaro | Studies on the non-pathogenicity of Chryseobacterim proteolyticurn and on the safety of the enzyme: Protein-glutaminase | 2007 | Regulatory Toxicology and Pharmacology | Volume 49, pgs 79-89 |

***NA- Not applicable***