Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include Protein-glutaminase from *Chryseobacterium proteolyticum* as a Processing Aid

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GENERAL REQUIREMENTS

1.0 APPLICANT DETAILS

a) [redacted]
b) Amano Enzyme Inc.
c) (Head office) 1-2-7, Nishiki, Naka-ku, Nagoya, Aichi 460-8630 Japan
d) [redacted]
e) [redacted]
f) Enzyme manufacturer
g) [redacted]

2.0 PURPOSE OF THE APPLICATION

The purpose of the application is to amend Schedule 18 of the Food Standards Code to permit the use of protein-glutaminase from *Chryseobacterium proteolyticum* as a processing aid.
Protein-glutaminase is for use as an enzyme to deamidate proteins in food. In other words, the enzyme can improve protein functionality in food.

3.0 JUSTIFICATION FOR THE APPLICATION

3.1.1 Regulatory Impact Information

3.1.1.1 Cost and Benefit of the Proposed Change

Cost and benefit to consumers;

The inclusion of protein-glutaminase derived from *Chryseobacterium proteolyticum* in the Australia New Zealand Food Standards Code as a processing aid will no cost or benefits to consumers associated with the inclusion of protein-glutaminase in the Schedule 18. The use of protein-glutaminase is one of a number of commercial methods available to improve protein functionality of certain food products. The availability of a range of food products is the same, irrespective of the method employed to achieve the results.

Cost and benefit to Industry;

It will allow producers to add a protein-glutaminase step to their production process. The use of protein-glutaminase has a lot of benefit in food processing. Protein-glutaminase catalyzes the

deamidation of glutaminyl residue in the substrate polypeptide, resulting in the conversion of glutaminyl residues and release of ammonia.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 protein-glutaminase is an increase of solubility, because the formed negative charge of glutamic acid would contribute the electrostatic repulsion of intermolecular proteins.

Cost and benefit to Government;

There will be no additional cost to the regulator if the processing aid is approved as the use of protein-glutaminase derived from *Chryseobacterium proteolyticum* and will not impact the regulation of these food products since processing aids are machinery in nature and their use is voluntary.

3.1.1.2 Impact on International Trade

The approval of protein-glutaminase from *Chryseobacterium proteolyticum* as a processing aid may, in the future, promote international trade and reduction of technical barriers to trade, while continuing to protect public health and safety.

4.0 INFORMATION TO SUPPORT THE APPLICATION

Sections A through F of this application contain detailed data that supports the quality, efficacy, and safety of protein-glutaminase from *Chryseobacterium proteolyticum* under the proposed conditions of use as a processing aid in Australia and New Zealand, as presented in accordance with the information requirements listed in Section 3.3.2 (Processing Aids) of the Food Standards Australia New Zealand (FSANZ) Application Handbook (FSANZ, 2016). The data pertaining to the protein-glutaminase from *Chryseobacterium proteolyticum* in this application is representative of the commercial product for which approval is being sought.

The information is provided in this application to enable the objectives specified in Section 18 of the FSANZ Act to be addressed as follows:

a) The protection of public health and safety: Information to support objective (a) is provided in Section C of the application, in which the safety of protein-glutaminase derived from *Chryseobacterium proteolyticum*, based on the available pre-clinical and human safety data, is discussed in detail.
b) The provision of adequate information relating to food to enable consumers to make informed choices: Data to support objective (b) are provided in Section F, in which the impact and purpose of protein-glutaminase are described in detail.
c) The prevention of misleading or deceptive conduct: Information supporting objective (c) is

provided in Section F, in which the consumer awareness and potential behaviour in response to products manufactured using protein-glutaminase are described in detail. This objective can also be further supported by human safety data contained in Section C.

Additionally, as per the FSANZ Application Handbook (FSANZ, 2016), any evidence that the food industry generally or other specific companies have an interest, in, or support, the proposed changes to the Code is mandatory for applications to change the Food Standards Code. As discussed in Section C, the use of protein-glutaminase derived from *Chryseobacterium proteolyticum* has a history of use in France and the US. It is expected that the introduction of protein-glutaminase derived from *Chryseobacterium proteolyticum* to the Australia/New Zealand market will be well received.

5.0 ASSESSMENT PROCEDURE

Amano Enzyme considers the most appropriate assessment procedure for the application herein, which relates to an amendment Schedule 18 of the Food Standards Code to include protein-glutaminase derived from *Chryseobacterium proteolyticum* as a processing aid, to be the General Procedure (Subdivision D), Cost Category Level 1 (up to 350 hours). This is based on the fact that FSANZ had approved the similar food enzymes; Transglutaninase (EC 2.3.2.13) and Glutaminase (EC 3.5.1.2), and that Amano Enzyme's protein-glutaminase product has already been approved and marketed in several other major jurisdictions for food uses that are similar to those proposed in Australia/New Zealand.

6.0 CONFIDENTIAL COMMERCIAL INFORMATION

None of the information presented in this application are considered to be confidential commercial information.

7.0 EXCLUSIVE CAPTURABLE COMMERCIAL BENEFIT (ECCB)

It is not anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the Food Standards Australia New Zealand (FSANZ) Act, which states:

> An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food regulatory measure under Section 22 if:
>
> a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that

would be prepared in relation to the application; and

b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application

8.0 INTERNATIONAL AND NATIONAL STANDARDS

The following national and international standards are relevant to the current application (See also C.1):

- Protein-glutaminase complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006). (See also A.5.1)
- Protein-glutaminase from *Chryseobacterium proteolyticum* is approved in France.
- FDA responded to the GRAS notification submitted by the Amano Enzyme Inc. that FDA has no questions regarding that protein-glutaminase enzyme preparation from *Chryseobacterium proteolyticum* is GRAS (GRAS Notice No. GRN 267).

9.0 STATUTORY DECLARATION

A signed statutory declaration is appended to this application.

10.0 CHECKLIST

A completed checklist relating to the information required for submission is appended to this application.

SECTION A: TECHNICAL DESCRIPTION OF Protein-glutaminase

Protein-glutaminase is an enzyme of microbial origin that is proposed for use as a processing aid in Australia and New Zealand. A full description of the processing aid including the identity, enzymatic properties, manufacturing process, and purity is presented in this section.

A.1 Information on the Type of Processing Aid

Protein-glutaminase is powdered enzyme and is a protein-deamidating enzyme produced from the microorganism *Chryseobacterium proteolyticum* isolated from a soil.
Protein-glutaminase catalyzes the deamidation of glutaminyl residues in the substrate polypeptide, resulting in the conversion of glutaminyl residues to glutamyl residues and release of ammonia.
The general reaction scheme is:

Protein L-glutamine + H_2O → Protein L-glutamate + NH_3

Protein-glutaminase catalyzed the deamidation of intact protein without any need of transglutaminase or protease activities.

Amano Enzyme has prepared protein-glutaminase enzyme preparation that is derived from *Chryseobacterium proteolyticum* by means of a fermentation process. The enzyme intended for use as a processing aid in food. A full description of the manufacturing procedures is provided in Section A.4.
Based on the foregoing description, protein-glutaminase from *Chryseobacterium proteolyticum* would fall under the following classification within Schedule 18 (Processing Aids):

18-4 (5) Permitted enzymes of microbial origin

The maximum proposed level of protein-glutaminase to food products use is 0.64%. (Normally the enzyme, protein-glutaminase is diluted with cassava dextrin (Protein-glutaminase: 75%, Dextrin: 25%). Therefore, the maximum proposed level of the enzyme preparation including dextrin to food products use is 0.85% (0.64/0.75)). (See also Section F.1)

A.2 Information on the Identity of the Processing Aid

Common name :	Protein-glutaminase
Systematic name:	protein-L-glutamine amidohydrolase
E.C. number:	3.5.1.44
CAS registry number:	62213-11-0

The protein-glutaminase is produced by *Chryseobacterium proteolyticum* AE-PG. Strain AE-PG is not genetically modified organism but a chemically mutated production strain derived from the original strain (See also section D.1). *Chryseobacterium proteolyticum* has been used for many years for food

or feedstuffs purposes or in the production of enzymes processing aids in the U.S. and France.

A.3 Information on the Chemical and Physical Properties of the Processing Aid

A.3.1 Technological Function and Enzymatic Properties

A.3.1.1 Assay for Measuring Enzyme Activity (Deamidating activity)

An analytical method for the detection and quantification of enzyme activity is presented in Appendix A - 1. Enzyme activity can be obtained by absorption photometry. One enzyme activity unit is defined as the quantity of enzyme that will produce 1μmol of ammonia per 1 minute under the conditions of the assay.

A.3.1.2 Characterization of Enzyme Activity (Deamidating Activity)

The technical function of protein-glutaminase is to catalyze the deamidation of glutaminyl residues in the substrate polypeptide into glutamyl residues and release of ammonia.

The effects of temperature and pH on the activity of the protein-glutaminase were examined and the results are presented in Figure A-1 and Figure A-2. In all assays the same experimental procedures described in Section A.3.1 were employed with the only modifications affecting the temperature of the water bath or the pH of the protein-glutaminase solution. The effect of temperature and pH on the activity were compared to the activity measured under standard conditions. For the assessment of the impact of temperature on activity, the standard conditions were considered to be a water bath temperature of 37°C. Based on the assays conducted, exhibits activity from pH 3.0 till pH 10.0, and from 20°C till 70°C.

Figure A - 1: Effect of pH

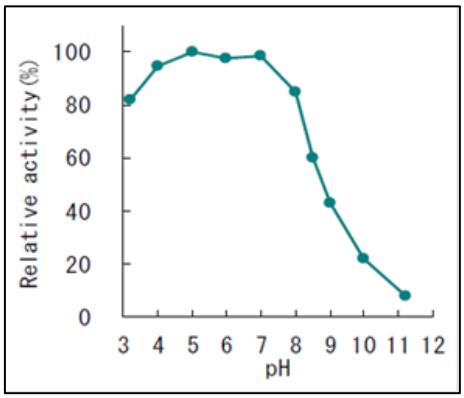
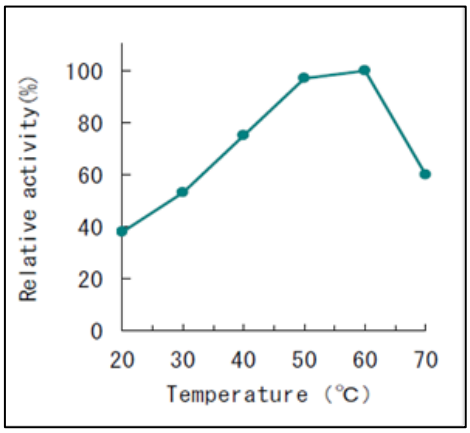


Figure A - 2: Effect of Temperature



A.3.2 Stability

pH and THERMAL STABILITY

The stability of protein-glutaminase has been assayed. As the enzyme activity was considered the primary marker of the stability of protein-glutaminase, the experimental procedures described in Section A.3.1 were employed to assess the stability. The only change to the experimental procedures was the duration of the incubation. The results of the assessment of the thermal and pH stability are presented in Figures A-3 and A-4.

The results of the assessment of stability under varying temperature and pH conditions indicate that protein-glutaminase is stable at 50-60°C and in a pH range of 5.0-7.0.

Figure A - 3: pH stability

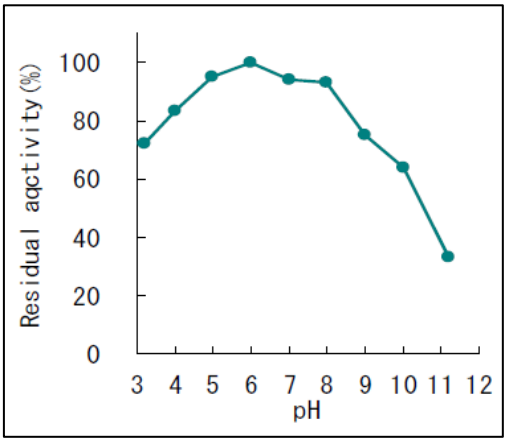
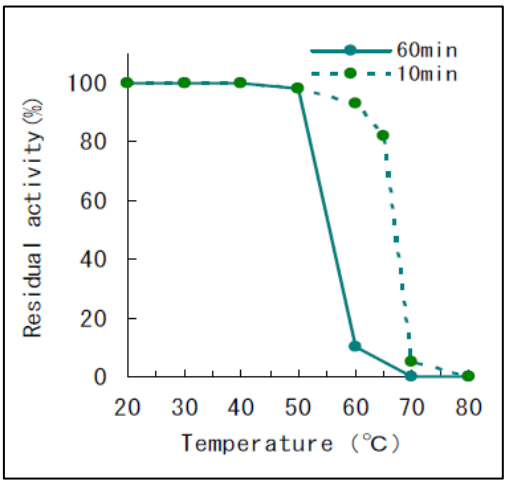


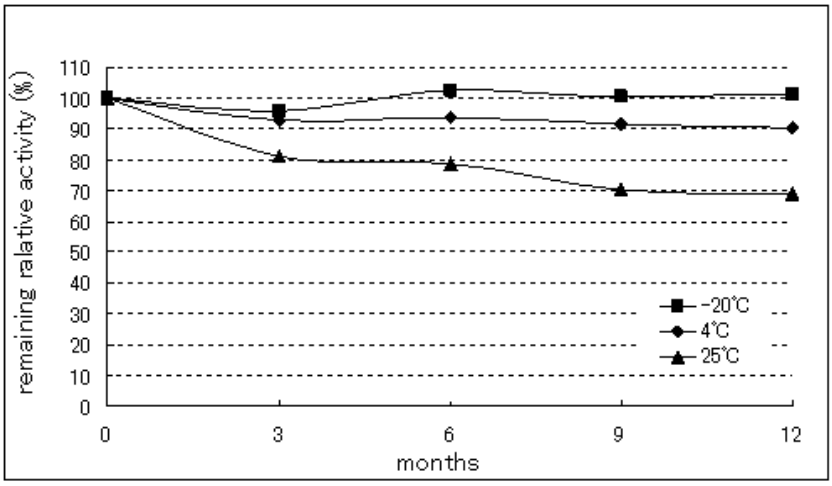
Figure A - 4: Thermal stability



LONG TERM STABILITY

The stability of the protein-glutaminase preparation was assayed by the Amano Enzyme Inc. Samples of the preparation were putted into an airtight bag and kept at -20°C, 4°C and 25°C. The deamidating activity was periodically measured for 12 months by the method indicated in the Section A.3.1. Results are summarized in Figure A-5.

Figure A - 5 : The stability of the enzyme preparation



A.3.3 Possible Interactions with Food Constituents

Protein-glutaminase is an enzyme which acts on single substrate and would therefore, not be expected to act on other constituents in the food. The enzyme preparation must be inactivated either by temperature or pH changes. Amano Enzyme recommends that the inactivation be accomplished by increasing the temperature above 70°C. Food manufacturers conforming to the recommended conditions of use will ensure that the enzyme is inactivated in the final food product and therefore, unable to react with any glutaminyl residues present in non-target foods.

A.3.4 Characterisation of Secondary Activities

As far as Amano is aware, the protein-glutaminase described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

Microbial food enzymes are concentrates typically containing minor amounts of other enzyme activities (side activities) naturally produced by the microorganism. However, these activities are not relevant from an application or safety point of view, even if it concerns proteases and phospholipases.

Proteases and phospholipases, like many other enzymes, are widely sold as digestive aids, both as over-the-counter registered pharmaceutical products and as dietary supplements. Some of these are available even as chewable dietary supplements. No effects on mucous membranes have been reported, although the enzymes in digestive aids are ingested in their active form and the oral exposure is orders of magnitude higher than the insignificant exposure from food enzymes used as processing aids in food manufacturing.

Furthermore, a wide range of food enzymes, including proteases and phospholipases, have been on the market for decades and have been approved on the market for use in food on basis of safety documentation.

Finally proteases and phospholipases are natural constituents of foods. For instance, bromelain is a protease that is ingested in its active form by consumers eating raw pineapples. Phospholipase is a normal constituent of wheat flour (Nolte et al., 1974) and is one of the digestive enzymes present in the pancreatic juice of mammals, including humans (de Haas et al., 1968; Rossiter, 1968; Johnson and McDermott, 1974).

In order to demonstrate above, the analyses were performed for amylase activity (Starch dextrinizing activity), lipase activity and protease activity. The results are summarized in the Table below and Appendix A - 2.

Table A - 1: Side activity in the enzyme preparation

		batches		
Activities (u/g)	Method	PGG0352501K	PGF0451601K	PGE0952601K
Lipase activity	JP method *	Not detected	Not detected	Not detected
Amylase activity	Amano method	Not detected	Not detected	Not detected
Protease activity	Amano method	8.3	8.7	8.4
Deamidating activity	Amano method	537	520	565

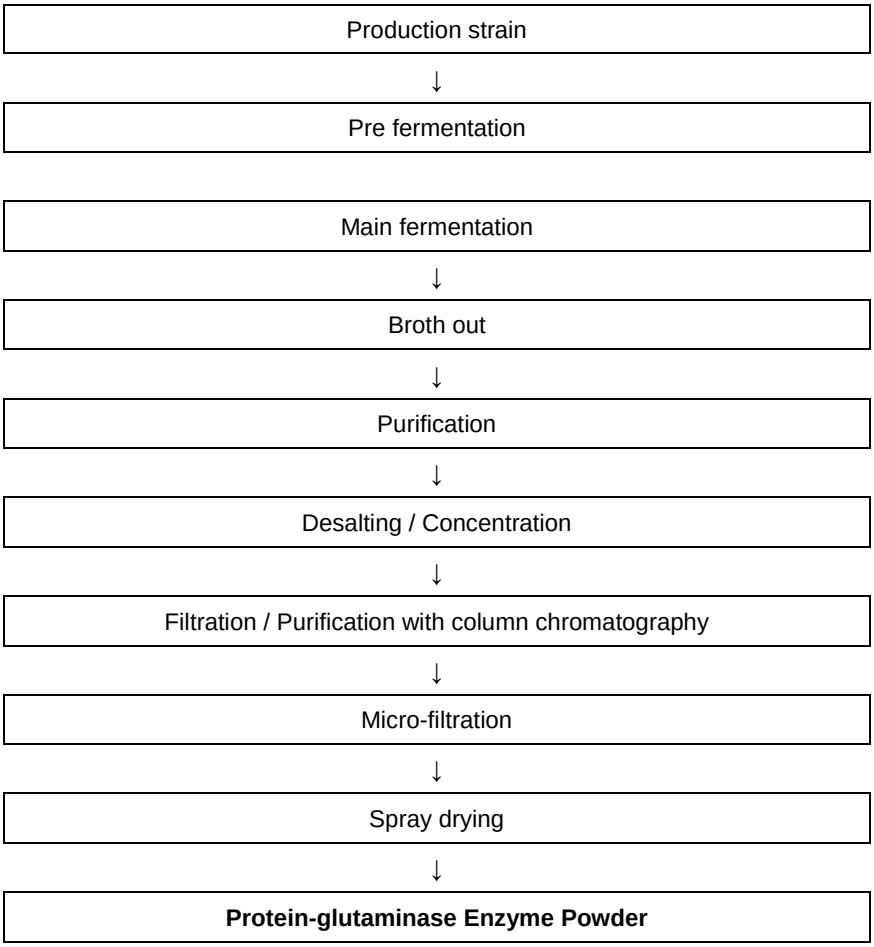
* Japanese pharmacopeia

A.4 Manufacturing Process

A.4.1 Manufacturing Steps

A schematic overview of the overall manufacturing process for protein-glutaminase is provided in Figure below.

Figure A - 6: Manufacturing Process



In brief, the production begins with the fermentation of *Chryseobacterium proteolyticum* under standard culturing conditions. Recombinant DNA technology is not used to obtain this strain. Once the fermentation is complete, the broth is then submitted to a series of separation and concentration steps at the end of which the food enzyme concentrate can be formulated into a commercial preparation that will be used in food processing. Normally, protein-glutaminase enzyme powder is diluted with dextrin to apply for food processing (Protein-glutaminase 75%, Dextrin 25%). The proposed amount of protein-glutaminase to show the function in various foods is indicated in the section F.1.

The enzyme preparation is produced according to the FSSC22000 food safety system and complies with international guidelines for the safe handling of microbial enzyme preparations published by the Association of Manufacturers of Fermentation Enzyme Products (AMFEP).

The Good Manufacturing Practices (GMP) for food additives certification and certificate of conformity to FSSC22000 are provided in Appendix A - 3.

A.4.2 Raw Materials

The raw materials employed in the production of protein-glutaminase is listed in following table along with the grade of material employed, the function in the production process, and the status of the raw material in Australia and New Zealand. All of the raw materials employed in the production of protein-glutaminase enzyme are of appropriate quality for use in foods. The raw materials are all approved for use in the food supply in Australia and New Zealand either as food ingredients, raw materials in used in the production of processing aids or foods additives, or as food additives themselves.

Table A - 2: Raw Materials and Processing aids used for the production

Raw Materials and Processing aids Used in the Production			
Substance	**Grade**	**Function**	**Status in Australia and New Zealand (FSANZ, 2014)**
Soybean oil	Food	Culture media	Food ingredients
Glucose	Food	Culture media	Food ingredients
Yeast extract	Food	Culture media, Processing	Food ingredients
Yeast peptide	Food	Culture media	Food ingredients
Soy peptone	Food	Culture media	Food ingredients
Potato starch	Food	Culture media, Processing	Food ingredients
Glutamine peptide	Food	Culture media	Food ingredients
Gluten meal	Food	Culture media	Food ingredients
Potassium phosphate, dibasic	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Potassium phosphate, monobasic	Food additive	Culture media, Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Magnesium sulphate	Food additive	Culture media	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Acetic acid	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Calcium chloride	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Diatomaceous earth	Food additive	Processing	Permitted for use in the production of processing aids (Schedule 18)
Sodium acetate	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Sodium chloride	Food	Processing	Food ingredients
Sodium hydroxide	Food additive	pH adjustment	Permitted for use in the production of processing aids (Schedule 18)
Sodium sulphate	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)

Raw Materials and Processing aids Used in the Production			
Substance	**Grade**	**Function**	**Status in Australia and New Zealand (FSANZ, 2014)**
Sodium phosphate, d basic	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Acid clays of montmorillonite	Food additive	Processing	Permitted for use in the production of processing aids (Schedule 18)
Magnesium chloride	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Calcium disodium EDTA	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
L-Cystein monohydrochloride	Food additive	Processing	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Cassava dextrin	Food	Processing, Diluent	Food ingredients

A.4.3 Residual Allergens from the Culture Medium

Soybean derivatives are used as fermentation media. The residual amount was tested and not detected in the protein-glutaminase enzyme powder (Detection limit: 1.0ppm). As described in section A.1, normally, protein-glutaminase enzyme powder is diluted with dextrin (25% dextrin). Therefore, the residual amount of soy protein in final enzyme preparation could be calculated at 0.75ppm or less.

A.5 Specification for Identity and Purity

A.5.1 Product Specification

The Chemical and Microbiological Specification

It is proposed that the food enzyme protein-glutaminase should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006):

Table A - 3: Specification for Protein-glutaminase

The Chemical and Microbiological Specification	
Lead	Not more than 5 mg/kg
Salmonella sp.	Absent in 25 g of sample
Total coliforms	Not more than 30 per gram
Escherichia coli	Absent in 25 g of sample
Antimicrobial activity	Not detected
Mycotoxins	Not applicable for bacterial enzymes
Enzyme Activity	
Activity (Deamidating activity)	Not less than 500 u/g
General Properties	
Appearance	Light yellowish white powder

A.5.2 Batch Analysis

The proof that the food enzyme protein-glutaminase complies with these specifications is shown by the analyses on various different batches, see Appendix A - 4.

Protein content and relative purity of the food enzyme protein-glutaminase from *Chryseobacterium proteolyticum* was measured, and the TOS values were calculated, in 3 batches. The result is shown in the following Table.

Table A - 4: Batch Analysis (Protein-glutaminase Enzyme Powder)

Batch no	**PG(SDY)-Y59 -001$K**	**PG(SDY)-Y61 -001$K**	**PG2(SDY)-Y66 -001$K**	**Mean**
Heavy metals				
Lead	0.01 mg/kg	0.03 mg/kg	0.01 mg/kg	-
Microbiology				
Salmonella sp.	ND/25g	ND/25g	ND/25g	-
Total coliforms	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g	-
Escherichia coli	ND/10g	ND/10g	ND/10g	-
Antimicrobial activity				
Antimicrobial	Negative	Negative	Negative	-
Protein content and relative purity				
Ash (%)	2.0	1.6	2.6	2.09
Water (%)	6.3	4.5	3.3	4.7
Carrier (%)	90.7	92.8	92.6	92.0
TOS (%)	1.0	1.1	1.5	**1.2**
Enzyme activity (u/g)	686	780	872	779
Units/mg TOS	68.6	70.9	58.1	65.9
Protein (%)	9.5	5.9	8.9	8.1

ABSENCE OF TOXINS

As the species Chryseobacterium proteolyticum is bacteria, it is not known to produce any bacterial toxins. However, to confirm the absence of any toxins, the analysis was conducted. The results are in the following table (Appendix A - 5). It can be concluded that *Chryseobacterium proteolyticum* has no safety concern.

Table A - 5: Search for Mycotoxins

Date of certificate	Mycotoxin	Result
29 July 2009	Aflatoxin B1	Not detected
	Aflatoxin B2	Not detected
	Aflatoxin G1	Not detected
	Aflatoxin G2	Not detected
	Ochratoxin A	Not detected
	Sterigmatocystin	Not detected
	Zearalenone	Not detected
	T-2 toxin	Not detected

A.6 Analytical Method for Detection

In accordance with section 3.3.2 of the FSANZ Application Handbook, an analytical method for detection is not required for an enzymatic processing aid (FSANZ, 2016). Therefore, this section is not relevant to the use of protein-glutaminase from *Chryseobacterium proteolyticum*.

SECTION B: INFORMATION RELATING TO THE SAFETY OF A CHEMICAL PROCESSING AID

This section is not relevant to the current processing aid and therefore is not included in this application.

SECTION C: INFORMATION RELATING TO THE SAFETY OF AN ENZYME PROCESSING AID

C.1 General Information on the Use of the Enzyme as a Food Processing Aid in Other Countries

- Protein-glutaminase from *Chryseobacterium proteolyticum* is approval in France[1]. (Appendix C - 1)

- FDA responded to the GRAS notification submitted by the Amano Enzyme Inc. that FDA has no questions regarding that protein-glutaminase enzyme preparation from *Chryseobacterium proteolyticum* is GRAS (GRAS Notice No. GRN 267). (Appendix C - 2)

C.2 Information on the Potential Toxicity of the Enzyme Processing Aid

As mentioned in Section C.1, protein-glutaminase has a wide history of use in food processing. To further support the safety of protein-glutaminase enzyme preparation, several toxicity studies have been conducted to assess the safety. The potential mutagenic and genotoxic activity of the protein-glutaminase were conducted through in vitro assessment, as well as a repeat-dose 13-week oral toxicity study conducted in rats. These studies are described below in Section C.2.1.
The food enzyme has been subjected to a standard package of toxicological tests, with the following results:

- Bacterial reverse mutation: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test conditions
- 90-day oral toxicity on rats: The No Observed Adverse Effect Level (NOAEL) is 46.4 mg TOS/kg bw/day, which is the middle dose in the study.

[1] https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000271061&dateTexte=20160822

C.2.1 Protein-glutaminase

C.2.1.1 Mutagenicity and Genotoxicity

The following two genotoxicity studies and a chronic toxicity study were carried out in accordance with Japanese recognized guidelines.

A bacterial reverse mutation test (Appendix C - 3)

Reference:

Sui H. (2004) Safety study of protein glutaminase produced by *Chryseobacterium proteolyticum* – reverse mutation test in bacteria. Hatano Research Institute, Food and Drug Safety center. Project No.: M-03-067.

Summary:

A reversed mutation test using *Salmonella typhimurium* and *Escherichia coli* was carried out in 2004 by the Hatano Research Institute, according to the guidelines of OECD and Ministry of Health and Welfare (Japan).

Four *Salmonella typhimurium* strains (TA98, TA100, TA1535 and TA1537) and one *Escherichia coli* strain (WP2uvrA) were assayed with and without metabolic activation (S9 mix).

This assay complies with Good Laboratories Practices (GLP) and Quality Assurance (QA).

The test substance was a concentrate of protein-glutaminase derived from *Chryseobacterium proteolyticum* (Batch No.: PGP2-030930, Protein-glutaminase activity: 1024 u/g). For the dose-finding test, the doses ranged from 50 to 5,000 µg/plate with or without S9 mix. The final test was duplicated with doses ranging from 313 to 5,000 µg/plate with or without S9 mix.

Recommended positive controls were assayed.

The results of this study indicate that the test article did neither inhibit the growth of the bacteria at doses up to 5,000 µg/plate nor show mutagenic effects in the *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537) or in *Escherichia coli* (WP2uvrA) with or without metabolic activation.

Chromosome aberration test (Appendix C - 4)

Reference:

Yamakage K. (2004) Safety study of protein-glutaminase produced by *Chryseobacterium proteolyticum* – Chromosomal aberration test using Chinese hamster lung (CHL/IU) cells. Hatano Research Institute, Food and Drug Safety center. Project No.: G-03-046.

Summary:

A mammalian cells chromosomal aberration study of protein-glutaminase was carried out in 2004 by the Hatano Research Institute, according to the guidelines of OECD and Ministry of Health and Welfare (Japan).

CHL/IU cells from Chinese hamster lung were assayed with and without metabolic activation (S9 mix).

This assay complies with Good Laboratories Practices (GLP) and Quality Assurance (QA).

The test substance was a concentrate of protein-glutaminase derived from *Chryseobacterium proteolyticum* (batch No.: PGP2-030930, 1024 U/g).

During the growth inhibition test, up to 5,000 µg/ml was assayed with and without S9 mix. Protein-glutaminase showed cell growth inhibition in all treatment system. The ID50 was estimated to be 820, 460 or 250 µg/ml for short-term treatment without S9 mix, for short-term treatment with S9 mix, and for 24 h continuous treatment, respectively.

For the cytogenetic test, up to 1700, 850 and 500 µg/ml were assayed for short-term treatments without S9 mix and with S9 mix, and for 24 h continuous treatment, respectively. Classical positive controls were performed.

Chromosomal aberrations (structural chromosome aberrations and polyploid cells) were not induced at any doses in the presence or not of metabolic activation. It was concluded that protein-glutaminase concentrate was not clastogenic.

C.2.1.2 Repeat Dose Toxicity Assay

Characterization of the samples assayed for subchronic toxicity

Analyses of the test article (Batch PG-Y57-002@) assayed for repeated toxicity testing are provided in the attached data 1 (page 31) of the 13 week toxicity study report. These data have been completed by Amano on May 2008, in order to define the TOS of this batch (Appendix C - 5).

In summary, the test article has the following characteristics:

- Lot number: PG-Y57-002@
- Specific activity: 549 U/ml
- TOS : 0.928%

For this study, the protein-glutaminase concentrate has been diluted 3.6 times to reach a maximum concentration to be assayed of 253.8 mg/ml.

Sub-chronic toxicity (13-week oral toxicity study) (Appendix C - 6)

Reference:

Yamaguchi T. (2005) A 13-week oral toxicity study of protein-glutaminase in rats. Bozo Research Center, study No.: B-5339.

Summary:

A 13 weeks subchronic oral toxicity study (by gavage) was performed in 2004-2005, by the Bozo Research Center (Tokyo, Japan).

The study was performed according to the Ministry of Health and Welfare (Japan) guidelines for the testing of food additives.

This study complies with the principles of Good Laboratory Practices and Quality Assurance.

The study was carried out in 6 weeks old Sprague Dawley SPF rats using a protein-glutaminase liquid concentrate (Lot No.: PG-Y57-002@, 549 u/mL) by oral (gavage) administration.

Three groups, each comprising 12 males and 12 females, received the test material at doses of 25%, 50% and 100% solution, each volume at 10.0 ml/kg/day. There were no test article-related deaths in any group. Administration of the test article was associated with no effects in clinical signs, body weight, food consumption, ophthalmology, haematology, organ weight, necropsy or histopathological examination.

In urinalysis, a physiological change was observed. However, in the test result were judged to be within the range of physiological variations since there were no animals which showed significant deviations from the background data of the testing facility.

In hematology, prolongation of prothrombin time and a low fibrinogen value with statistical significance was observed in the high dose group. These were physiological changes because these were within the deviations from the background data of the testing facility in the report. However, it is thought that these changes should not neglect for the evaluation of the safety of the test material. Therefore, the no observed adverse effect level of the test material was selected at the middle dose (50% solution group,

correspond to 46.4 mgTOS/kg/day).

Result:

For this study, the protein-glutaminase concentrate has been diluted 3.6 times to reach a maximum concentration to be assayed of 253.8 mg/ml.

Considering that 10 ml/kg body weight of this solution were administered (Table 1 page 15 of the report), considering a specific gravity of 1, the maximal assayed concentration expressed in TROS is:

10 x 0.928% = 92.8 mg TOS/kg BW

As described in the summary, the NOAEL is set at the middle dose (50% solution group).

The NOAEL was determined to be 1269mg PG preparation/kg BW/day (2538/2) corresponding to 46.4 mg TOS/kg BW/day (92.8/2)

SAFETY MARGIN

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). As was shown in Section F.3, the Total TMDI of the food enzyme is 0.38 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 46.4 / 0.38 = 122

As is explained in Section F.3, the Total TMDI is highly exaggerated. Therefore, the actual MoS in practice will be higher. Consequently, there are no safety reasons for laying down maximum levels of use.

C.3 Information on Potential Allergenicity

C.3.1 Source of the Processing Aid

Chryseobacterium proteolyticum is used and approved for use as a source organism in France and the US (refer to the section 8.0). No allergenicity warnings are associated with the use of this organism in food in these countries. Amano's *Chryseobacterium proteolyticum* in this submission has been sold and used safely for the production of food enzymes for around 20 years. Meanwhile no pathogenic or toxic accident has been arisen in the workers exposed to the strain. Furthermore, it has a long history of use in food industry (see the section C.3.2: long history of use).

C.3.2 Allergenicity of Protein-glutaminase

Amino-acid sequence

Amino-acid sequence of protein-glutaminase was determined. The results of homology search by matching for 8-consecutive amino acid sequence and 80 amino acid sliding window searches13 indicated that the coincidence with known allergen was not observed (Appendix C - 7).

Literature Search

In order to address allergenicity by ingestion, it may be taken into account that:

- The allergenic potential of enzymes was studied by Bindslev-Jensen et al. (2006) and reported in the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Dauvrin et al., 1998). The overall conclusion is that exposure to enzyme proteins by ingestion, as opposed to exposure by inhalation, are not potent allergens and that sensitization to ingested enzymes is rare.

- Enzymes when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.

Thus, there are no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers.

Additional considerations supporting the assumptions that the ingestion of an enzyme protein is not a concern for food allergy should also be taken into account:

- The majority of proteins are not food allergens and based on previous experience, the enzyme industry is not aware of any enzyme proteins used in food that are homologous to known food allergens.
- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al., 2008).
- In the case where proteins are denatured, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft, 2002; Valenta, 2002; Takai et al., 1997; Takai et al., 2000; Nakazawa et al., 2005; Kikuchi et al., 2006).
- In addition, residual enzyme still present in the final food will be subjected to digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001[2]; Goodman et al., 2008).
- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

[2] http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf

Long History of Use

Since isolated in 1997 to date, this strain has been handled at our R&D center. And the protein-glutaminase preparation has been produced at Amano and used in food industry area. During that period, no safety concerns have been reported. Therefore, it can be said that protein-glutaminase from *Chryseobacterium proteolyticum* has a long history of sage use.

C.4 Safety Assessment Reports Prepared by International Agencies or other National Government Agencies

- Protein-glutaminase from *Chryseobacterium proteolyticum* was evaluated in France (AFSSA). AFSSA concluded that the proposed uses did not present a health risk to the consumer and rendered a favorable opinion on its use.
- FDA responded to the GRAS notification submitted by the Amano Enzyme Inc. that FDA has no questions regarding that protein-glutaminase enzyme preparation from *Chryseobacterium proteolyticum* is GRAS (Appendix C - 8).

SECTION D: ADDITIONAL INFORMATION RELATED TO THE SAFETY OF THE ENZYME PROCESSING AID

D.1 Information on the Source Microorganism

General Information

The production organism for this enzyme preparation is a strain of *Chryseobacterium proteolyticum*. The wild type strain, *Chryseobacterium proteolyticum*, is common and widely distributed in soil.
Amano's *Chryseobacterium proteolyticum* has been used safely for the production of food enzymes for many years.

The production strain AE-PG was obtained by several mutations of the original strain that was found in Japanese soil. The production strain is derived via selection by conventional mutagenesis using NTG (N-methyl-N'-nitro-N-nitrosoguanidine) (Appendix D - 1). Recombinant DNA technology is not used to obtain this strain. It has been identified as *Chryseobacterium proteolyticum*.

The microorganism that is used for the production of protein-glutaminase, is bacteria, *Chryseobacterium proteolyticum*. According to the current taxonomic classification, the microorganism is classified as follows:

Genus	*Chryseobacterium*
Species	*proteolyticum*

Identification of the source organism

The protein-Glutaminase producing parent strain, isolated from a soil, was taxonomically classified as *Chryseobacterium proteolyticum* by Yamaguchi and Yokoe (Appendix D - 2).
The genus *Chryseobacterium* has been characterized in 1994 by Vandamme et al.(2002). Several species belonging to this genus have been described in by Shimomura et al. (2005) and Kim et al. (2005). The details of this enzyme producing strain have been reported by Yamaguchi (Appendix D-2). As indicated in the Appendix D-2, protein-Glutaminase producing parent bacterium, is rod-shaped, nonmotile, and nonsporing. Gram staining is negative. The cells are 0.4 to 0.5 µm wide and 0.8 to 2.0 µm long. They are aerobic and positive for oxidase and catalase, producing an insoluble yellow or orange pigment, which turned red with 3% KOH and returned to orange by neutralization, indicating a flexirubin type of pigment. These phenotypic characterization of the isolates indicate that it is included in the genus *Chryseobacterium*, which belongs to the family *Flavobacteriaceae*. However, no given strain is coinciding in acid formation from sucrose on the differential characteristics studies, therefore, the new isolates should be placed as a new species in the genus *Chryseobacterium*.
The strain has been deposited in the Patent Microorganism Depository, National Institute of Bioscience and Human Technology as strain FERM P-17664.

History of Use

The strain *Chryseobacterium proteolyticum* has been used safely for around 20 years to produce protein-Glutaminase preparation in Japan.

No other histories of food uses are known.

However, it is reported that *Chryseobacterium balustinum*, a phylogenic close strain to *Chryseobacterium proteolyticum*, was used as a protease producing bacterium for a cheese flavor (Garcia-Lopez et al. 2000). In addition, it is also reported that a number of strains classified as Chryseobacterium sp. can be found in dairy foods (Shimomura et al. 2005, Jooste & Hugo 1999).

D.2 Information on the Pathogenicity and Toxicity of the Source Microorganism

As described in the section C.3.2, the enzyme has a long history of safe use in food processing. No adverse effects have been reported in worker exposed to the source strain or enzyme preparation, or consumer so far.

Risk group evaluation is not available by any culture collections for *C. proteolyticum* since which has not been deposited at any official culture collections. However, all isolates from soil, waste water, sludge, and plant roots, other than those from clinical specimen are classified into risk group 1 in DSMZ and ATCC. This species, isolated from soil, is most likely to be as Risk group 1.

With reference to the Risk Classification of the European Community (Directive 2000/54/EC) *C. proteolyticum* could be classified in Group 1: biological agent that is most unlikely to cause human disease (Appendix D - 3).

The pathogenicity and toxinogenicity of *Chryseobacterium proteolyticum* has been investigated by Mizutani et al. (2000) and the data have been summarized and published by Scheuplein et al. (2007). Moreover, an additionally conducted test about localized, focal necrosis in liver has been investigated by Yokochi (2008).

The experimental design is presented in the following Table.

Table D - 1: Experimental design of single intravenous and oral inoculation studies

Groups	Inoculation volume (ml/mouse)	Number of animals
Single intravenous inoculation		
Saline control	0.1	5
C. proteolyticum		
2.9×10^7 CFU/mouse	0.1	10
2.9×10^8 CFU/mouse	0.1	10
Supernatant of culture broth	0.1	10
Supernatant of cell lysate	0.1	10
Single oral inoculation		
Saline control	0.5	5
C. proteolyticum		
1.3×10^9 CFU/mouse	0.5	10

No death occurred by intravenous or oral administration of *Chryseobacterium proteolyticum*. No changes in body weight were observed. No clinical signs were reported except transient locomotor activity, prone position and piloerection for the highest dose by intravenous administration.

Neither viable cells nor inflammatory signs were observed in any of the examined tissues (brain, lung, liver; spleen and kidney) following both oral and intravenous administration.

Histophatological examination of the previous tissues did not reveal any dose related effect. Only focal necrosis in the liver were observed at in the highest dose group for 4/10 animals of the intravenous administration study and for 1/5 animal of the oral administration one.

In comparison, positive controls using *P. aeruginosa* demonstrated body weight reduction, mortality, clinical signs, dose-related viable cells counts and histopathological changes.

Endotoxin concentrations in the supernatants of cell culture and lysate of the *C. proteolyticum* cells were both calculated to be 3 EU/mL. From this concentration, endotoxin dose in the cell suspensions was calculated to be 25 nglkg, which more than 6 times lower than the LD50 for mice.

In the above pathogenicity test, localized, focal necrosis in liver was observed in the venous inoculation in mice of the suspension of viable cells. However, an additionally conducted test using a non-pathogenic lactic acid bacterium as a control (Pariza & Johnson; 2001) indicated that the focal necrosis similar to the one that had been recognized by the producing bacteria was induced by the lactic acid bacteria as well, and that such focal necrosis in liver usually occurred even by non-pathogenic bacteria.

Form these findings, it was concluded that PG producing strain *C. proteolyticum* is not pathogenic.

D.3 Information on the Genetic Stability of the Source Organism

The source micro-organism is neither genetically modified nor self-cloned. The production strain was established by a repeated mutation process from the original strain. (Mutagen: NTG (N-methyl-N'-nitro-N-nitrosoguanidine)) (Appendix D - 4 and Appendix D - 5)

In order to ensure the genetic stability of the enzyme, it is produced under well controlled manufacturing processes which are in compliance with AMFEP's guidelines for the safe handling of microbial enzyme preparations (see Section A.4.1).

In brief, to ensure the genetic stability of the source organism, the production strain is fermented and is divided into an ampule. They are kept at below -70°C in a locked freezer.

When ready, an ampule is used for individual fermentation and after use, the residue is inactivated prior to discarding the vial. During fermentation the genetic stability of the source organism is monitored through the changes in pH and growth rates. In any instance where a deviation from normal is detected in either of these parameters, the fermentation media is removed from production and discarded. The strain is then checked to ensure that no genetic drift has occurred.

SECTION E: INFORMATION RELATING TO THE SAFETY OF AN ENZYME PROCESSING AID DERIVED FROM A GENETICALLY MODIFIED MICROORGANISM

This section is not relevant to the current processing aid and therefore is not included in this application.

SECTION F: INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE ENZYME PROCESSING AID

A summary of the proposed food uses, the anticipated residue level in foods, the anticipated exposure, and anticipated market share are presented in the Section below.

F.1 Proposed Food Uses

The technical function of protein-glutaminase is to catalyze the deamidation of glutaminyl residues in the substrate polypeptide into glutamyl residues and release of ammonia.
Protein-glutaminase will be used in variety of food manufacturing processes including:

- Baking and pasta / noodle making
- Milk and dairy processing
- Meat and fish processing
- Protein processing
- Grain processing
- Yeast processing

Food enzyme preparations are used by food manufacturers according to the *Quantum Satis* principle, which means that food manufacturers will typically fine-tune the enzyme dosage based on a dose range recommended by the enzyme supplier.
The table below provides recommended dose ranges in the various food processes:

Table F - 1: Recommended Dose Ranges

Application	**Raw material (RM)**	**Recommended use levels (mg TOS/kg RM)**
Baking and pasta / noodle making	Flour	1.5 - 15.3
Milk and dairy processing	Dairy products	0.08 - 0.8 (solid foods) 0.38 - 7.7 (beverages)
Meat and fish processing	Meat and Fish meat	3.8 - 15.3
Protein processing	Food derived proteins such as meat, egg, milk, soy, maize etc.	0.8 - 76.7
Grain processing	Cereal flours derived from wheat, oat, barley, soy, maize etc.	1.5 - 15.3 (solid foods) 0.2 – 2.7 (beverages)
Yeast processing	Yeast	1.0 – 6.5

Doses are expressed in Total Organic Solids (TOS).

The maximum proposed level of the enzyme preparation including diluent (dextrin) to food products is 0.85% in food processing. (As indicated above, the maximum use level of protein-glutaminase is 76.7 mgTOS/kg RM. TOS of protein-glutaminase enzyme powder is 1.2% (see the section A.5.2).) 0.85% is calculated by the following calculations:

76.7/ (0.012×1000) = 6.39 g/kg (0.012: TOS of enzyme powder is 1.2%)

6.39/0.75 = 8.5 g/kg = 0.85% (0.75: 75% of enzyme powder in final enzyme preparation)

F.2 Anticipated Residue Levels of Protein-glutaminase

The recommended use levels of the enzyme, protein-glutaminase are given based on the raw materials used in the various food processes.

Table F - 2: Maximal level in final food

Application		Raw material (RM)	Maximal recom-mended use level (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Beverages	Protein processing	Food derived proteins such as meat, egg, milk, soy, maize etc.	76.7	Soft drinks	0.05	3.8
	Dairy processing	Milk	7.7	Milk beverages etc.	1.0	**7.7**
	Grain processing	Grain drink	2.7	Oat milk etc.	1.0	2.7
Solid food	Baking and pasta/noodle making	Flour	15.3	Bread, pastry, cake, biscuit, cookie, pasta, noodle etc.	0.71	10.9
	Dairy processing	Milk	0.8	Cheese, yoghurt, ice cream etc.	10.0	8.0
	Meat and fish processing	Meat and Fish meat	15.3	Processed meat product such as sausage, ham etc. Fish-paste product	1.0	**15.3**
	Protein processing	Food derived proteins such as meat, egg, milk, soy, maize etc.	76.7	Sausage, ham, ground meat food, snacks, source, dressing, prepared food etc.	0.17	13.0
	Grain processing	Cereal flours derived from wheat, oat, barley, soy, maize etc.	15.3	Bread, pastry, cake, biscuit, cookie, pasta, noodle, snacks etc.	0.71	10.9
	Yeast processing	Yeast	6.5	Yeast products used in various solid foods	0.02	0.13

F.3 Information on the Likely Level of Consumption of Protein-glutaminase

The food enzyme is used in the manufacture of a wide variety of foods and food ingredients. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method Ref (Hansen, 1966; Douglass et al., 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data. The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed):

Average consumption over the course of a lifetime/kg body weight/day	Total solid food (kg)	Total non-milk beverages (l)	Processed food (50% of total solid food) (kg)	Soft drinks (25% of total beverages) (l)
	0.025	0.1	0.0125	0.025

The recommended use levels of the enzyme are given below based on the raw materials used in the various food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, it is assumed that all the TOS will end up in the final product.

Total TMDI

The Total TMDI can be calculated on basis of the maximal values found in food and beverage, multiplied by the average consumption of food and beverage/kg body weight/day. Consequently, the Total TMDI will be:

Table F - 3: The Total TMDI

TMDI in food (mg TOS/kg body weight/day)	**TMDI in beverage (mg TOS/kg body weight/day)**	**TMDI (mg TOS/kg body weight/day)**
15.3 x 0.0125 = 0.19	7.7 x 0.025 = 0.19	**0.38**

Based on the recommended use levels and the amounts of the respective ingredients that end up in the final foods, the TMDI of the food enzyme, protein-glutaminase is calculated to be:

0.38 mg TOS/kg body weight/day.

It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme protein-glutaminase from *Chryseobacterium proteolyticum*;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food as well as in beverage, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;
- Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al., 1997).

F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

There is no information on the expected use of this enzyme preparation in Australia/New Zealand or imported product currently sold in Australia/New Zealand.

F.5 Information relating to the levels of residues in foods in other countries

This enzyme is approved in France and the US. The approved food uses and maximum use levels are identical to those proposed for use in Australia. As a result it is anticipated that the levels of residues in foods imported from these jurisdictions would be identical to those manufactured in Australia.

F.6 For foods where consumption has changed in recent years, information on likely current food consumption

Not applicable.

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