FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

22 February 2018
[39-18]

Supporting document 1

Risk and technical assessment – Application A1136

Protein Glutaminase as a Processing Aid (Enzyme)

Executive summary

Amano Enzyme Inc. (Amano) has submitted an Application seeking permission to use an enzyme, protein glutaminase sourced from *Chryseobacterium proteolyticum,* as a processing aid in the manufacture of certain food products.

Protein glutaminase enhances protein solubility in various applications such as baking, pasta/noodle making, milk, dairy meat, fish, grain processing, yeast products and egg based products. The technological purpose is to improve emulsification, foam stabilisation and gelling in these proteinaceous foods. It also decreases flavour fade or 'off flavour' problems associated with flavour-protein interactions.

Enzymes used in the production and manufacture of food are considered processing aids and are regulated by Schedule 18 of the *Australia New Zealand Food Standards Code* (the Code). Permitted enzymes of microbial origin are listed in the Table to subsection S18—4(5) of the Code.

The safety data submitted by Amano in support of the Application was only for protein glutaminase sourced from one particular non-genetically modified strain of *Chryseobacterium proteolyticum* (i.e. *Chryseobacterium proteolyticum* strain AE-PG). FSANZ therefore only assessed protein glutaminase sourced from that particular strain.

There are no public health and safety issues associated with the use of protein glutaminase sourced from *Chryseobacterium proteolyticum* strain AE-PG as a food processing aid. Protein glutaminase was not genotoxic *in vitro* and did not cause adverse effects in a subchronic toxicity study in rats. In the absence of any identifiable hazard an Acceptable Daily Intake 'not specified' is appropriate. The enzyme does not have the characteristics of a potential food allergen.

FSANZ is satisfied that that enzyme's use as a processing aid in the manner specified in the Application is technologically justified. FSANZ also concludes that, as the enzyme performs its technological purpose during processing and manufacture of food only, it is appropriately categorised as a processing aid rather than a food additive. The proposed maximum permitted level is GMP (Good Manufacturing Practice).

The enzyme also complies with the internationally accepted Joint Expert Committee on Food Additives (JECFA) specifications for chemical and microbiological purity.

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1 Introduction and description

Amano Enzyme Inc. (Amano) is proposing to amend Schedule 18 of the *Australia New Zealand Food Standards Code* (the Code) to include protein glutaminase from a strain of *Chryseobacterium proteolyticum* as an enzyme of microbial origin. The enzyme is derived from a non-genetically modified strain of *C. proteolyticum*, with the production strain obtained by several mutations from the original strain found in Japanese soil.

Protein glutaminase (EC 3.5.1.44) catalyses the deamidation of glutaminyl residues in a substrate polypeptide. Deamidation is the removal or conversion of an amide functional group from the side chain of glutamine and results in the conversion of glutaminyl residues to glutamyl residues and release of ammonia.

The main benefit to food manufacturers using protein glutaminase is its ability to increase the solubility of proteins due to the formation of negatively charged glutamyl residues on the protein, which contributes to the electrostatic repulsion of intermolecular proteins. This results in protein-containing foods with enhanced protein solubility and potentially reduced "off flavour" problems compared to alternative chemical or heat treatments.

The functionality that protein glutaminase provides food manufacturers includes emulsification, foam stabilisation and gelling.

This enzyme is suitable for use in various food processing applications, such as baking and pasta/noodle making and the processing of milk, dairy, meat, fish, grain, yeast products and egg based products.

Protein glutaminase will be used as a processing aid in food manufacturing at *Quantum Satis* levels, with Amano providing indicative usage rates for various food matrixes.

Protein glutaminase is approved in France (2006) and the USA (2009), where the United States Food & Drug Administration (USFDA) responded to the Generally Recognized as Safe (GRAS) notification submitted by Amano with "no questions regarding protein glutaminase enzyme preparation from *Chryseobacterium proteolyticum*" (GRAS Notice No. GRN 267).

Protein glutaminase complies with the internationally accepted Joint Expert Committee on Food Additives (JECFA) specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006).

1.1 Identity

Information regarding the identity of the enzyme included in the Application has been verified using an appropriate enzyme nomenclature reference (IUBMB 2016).

Common name: Protein glutaminase

Other names: Protein-glutamine glutaminase, peptidoglutaminase II; glutaminyl-peptide glutaminase; destabilase; peptidylglutaminase II

Systematic name: protein-L-glutamine amidohydrolase

IUBMB[1] Enzyme Nomenclature: 3.5.1.44

E.C. number: 3.5.1.44

CAS registry number: 62213-11-0

Reaction catalysed:

Protein L-glutamine + H_2O <=> protein L-glutamate + NH_3

Protein glutaminase specifically hydrolyses the gamma-amide of glutamine substituted at the carboxyl position or both the alpha-amino and carboxyl positions, e.g., L-glutaminylglycine and L-phenylalanyl-L- glutaminylglycine.

The Amano protein glutaminase is produced from *C. proteolyticum* strain AE-PG. This strain is a non-genetically modified, chemically mutated production strain derived from the original strain (refer to section D.1 of Application). *C. proteolyticum* has been used for many years for food or feedstuffs, or in the production of enzyme processing aids in France and the U.S.

Furthermore, the Amano product specification, along with extensive batch analysis of protein glutaminase, demonstrate the purity of the enzyme preparation. This includes the absence of microbiological and metal contaminants, as well as the absence of antibiotic activity (to comply with international enzyme specifications relating to antimicrobial resistance concerns).

2 Food technology assessment

2.1 Technological purpose

The technical purpose of protein glutaminase is to catalyse the deamidation of glutaminyl residues in a substrate polypeptide into glutamyl residues, which also releases ammonia. This deamidation enables food manufacturers to increase the solubility of proteins, providing improvements in emulsification, foam stabilisation and gelling within a food matrix. The improved functional properties are due to the negative charge of the glutamyl residue contributing to the electrostatic repulsion of intermolecular proteins. Protein glutaminase is an enzyme that acts on a single substrate and would therefore not be expected to act on other constituents in the food.

Amano provided information demonstrating that the optimum pH range of the protein glutaminase deamidating activity is 5.0 – 7.0 as shown in Figure 1. Amano provided the analytical method for the detection and quantification of enzyme activity in Appendix A – 1 of the Application.

[1] International Union of Biochemistry and Molecular Biology

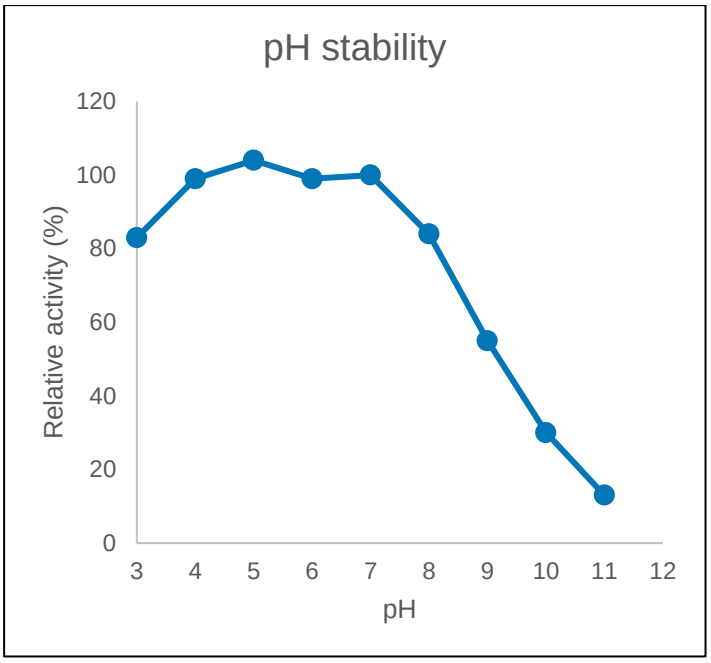

***Figure 1**. Effect of pH on enzyme activity*

The enzyme preparation is inactivated by adjusting either temperature or pH value. The optimum temperature for protein glutaminase functionality is 50 - 60°C, before inactivation occurs above 70°C (Figure 2).

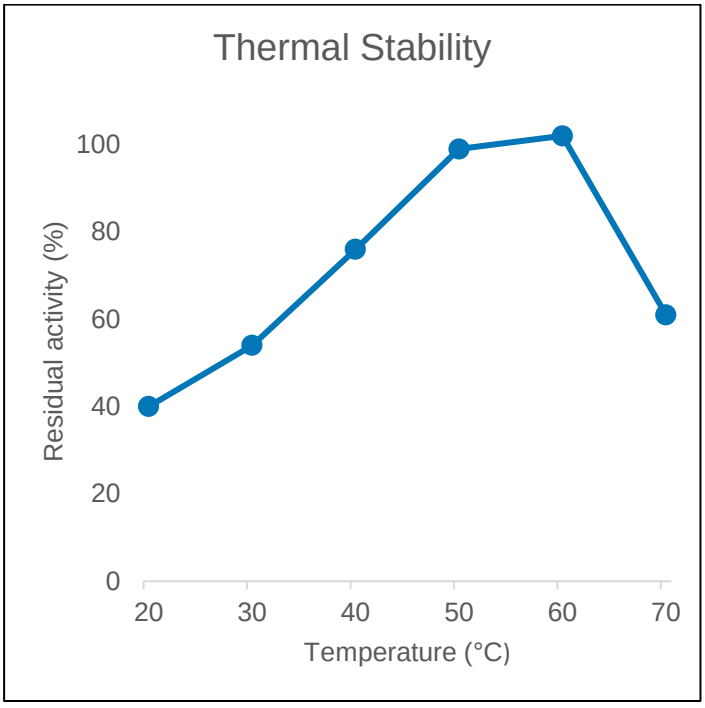

***Figure 2**. Effect of heat on enzyme activity*

Protein glutaminase meets the definition of a processing aid set out in the Code, which is that it performs a technological function during processing and does not perform a technological function in the food for sale.

2.2 Usage rates

Food enzymes are used by food manufacturers according to the *Quantum Satis* principle, which means that food manufacturers will typically fine-tune the usage rates based on a recommendation by the enzyme supplier. The Amano protein glutaminase enzyme powder is blended with Cassava dextrin to form an enzyme "preparation". This preparation is available in different grades, depending on the ratio of enzyme powder to dextrin. A 100% Protein glutaminase Enzyme Powder is also available. An example of one grade (75%) is as follows (w/w):

Protein glutaminase	75%
Cassava dextrin	25%
Enzyme "preparation"	100%

Recommended usage rates for Amano's 75% grade, enzyme "preparation" for various food manufacturing processes are provided in Table 1.

Table 1 Recommended enzyme "preparation" usage rates for 75% grade

Application	Raw material (RM)	Recommended usage rate (% w/w)	
		Min	Max
Baking and pasta / noodle making	Flour	0.017%	0.170%
Milk and dairy processing	Dairy products	0.001%	0.009% (solid foods)
		0.004%	0.086% (beverages)
Meat and fish processing	Meat and Fish meat	0.042%	0.170%
Protein processing	Food derived proteins such as meat, egg, milk, soy, maize etc.	0.009%	0.852%
Grain processing	Cereal flours derived from wheat, oat, barley, soy, maize etc.	0.017%	0.170% (solid foods)
		0.002%	0.030% (beverages)
Yeast processing	Yeast	0.011%	0.072%

2.3 Technological justification

Most plant proteins have poor solubility and therefore functionality under mild acidic conditions, which is the pH range of most food systems, resulting in their limited use in foods (Yamaguchi, 2000). Because the content of glutamine residue in plant proteins are generally high, deamidation of such proteins is regarded as a promising method to improve protein functionality in food systems (Yamaguchi, 2001).

In general, deamidated proteins have a decreased isoelectric point due to increased negatively charged carboxyl groups, resulting in a protein with increased solubility under more acidic conditions. This is particularly important for food proteins since many food matrixes are in the semi or high acid range.

Deamidation of proteins could also cause the alteration their tertiary structures. An unfolding of the protein would take place due to the electrostatic repulsion of newly formed negatively charged carboxyl groups. This unfolding leads to the exposure of hydrophobic regions, previously buried in the interior of the protein, to the aqueous surroundings. This alteration results in a protein with an improved amphiphilic character suited to use as an emulsifier, foaming or gelling agent.

Enzymatic methods for deamidation of proteins, such as the use of protein glutaminase, is selective, fast and occurs under mild reaction conditions within a food matrix (Hamada 1994). This is advantageous over other methods of protein deamidation such as thermal and/or chemical modification methods, which may produce undesired side effects on proteins, such as “off-flavour” formation (Yamaguchi, 2000).

FSANZ concludes that there is technological justification for the use of protein glutaminase in the applications suggest by Amano.

2.4 Manufacturing process

Protein glutaminase production begins with the fermentation of *C. proteolyticum* strain AE-PG under standard culturing conditions followed by several filtration and purification steps. The resulting protein glutaminase concentrate is then formulated into a commercial “enzyme preparation” for food manufacturers. Amano also have production controls in place to monitor the strain during the fermentation thereby ensuring no genetic drift.

The enzyme preparation is produced according to the FSSC22000 food safety system (for GMP certification) and complies with international guidelines for the safe handling of microbial enzyme preparations published by the Association of Manufacturers of Fermentation Enzyme Products. NTG (N-methyl-N'-nitro-N-nitrosoguanidine), a mutagen used to change the genetic material, was used to obtain the current production strain. Recombinant DNA technology was not used to obtain this strain and on that basis does not meet the legislative definition of a genetically modified organism.

The raw materials used in the production of protein glutaminase are safe and suitable for use and are permitted as either processing aids or foods additives in the Code. The finished Amano protein glutaminase enzyme is blended with a Cassava dextrin for use in food processing. The resultant enzyme preparation is a light yellowish-white powder.

2.5 Manufacturing flow chart

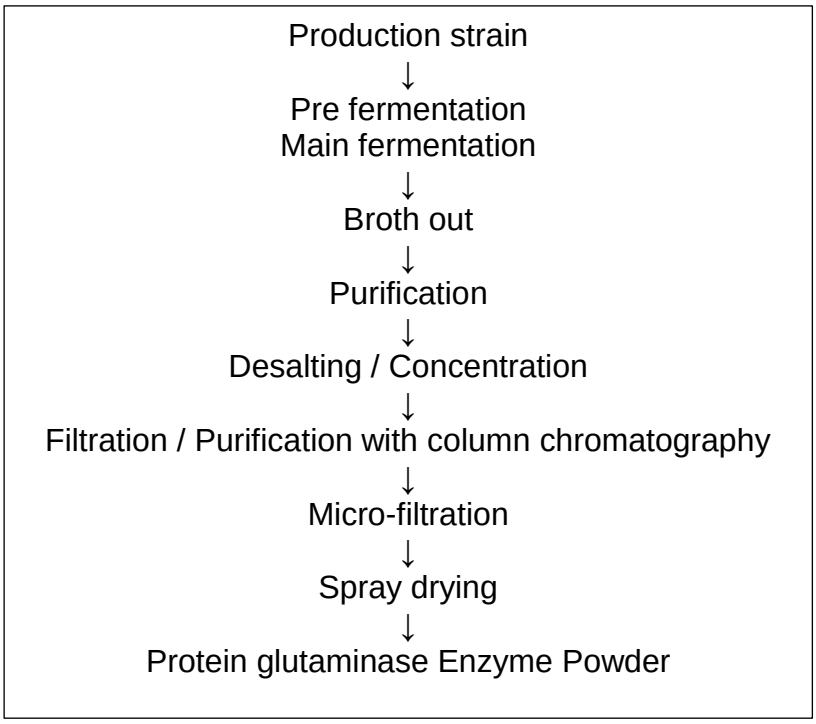

Fig 3: *Manufacturing Process for Protein glutaminase*

The Protein glutaminase Enzyme Powder blended with Cassava dextrin is available in different grades, depending on the ratio of enzyme powder to dextrin. A 100% Protein glutaminase Enzyme Powder is also available.

2.6 Product specification

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (JECFA 2016) and in the Food Chemicals Codex (Food Chemicals Codex 2014). These primary sources of specifications are listed in section S3—2 of Schedule 3 (Identity and Purity) of the Code. Enzyme preparations need to meet these enzyme specifications. Schedule 3 of the Code also includes specifications for heavy metals (section S3—4) if they are not included within specifications in sections S3—2 and S3—3.4.3 Chemical and Microbiological Specification. Table 2 shows the chemical and microbiological specifications for protein glutaminase, compared to three different production batches.

Table 2 Chemical and microbiological specification for protein glutaminase

	Specification	Batch 1	Batch 2	Batch 3
Metals				
Lead (mg/kg)	≤ 5	0.01	0.03	0.01
Microbiological				
Salmonella sp.	ND 25g	ND 25g	ND 25g	ND 25g
Total coliforms (cfu/g)	≤ 30	< 10	< 10	< 10
Escherichia coli	ND 25g	ND 10g	ND 10g	ND 10/g
Antimicrobial activity	Negative	Negative	Negative	Negative
Enzyme Activity				
Deamidating activity (u/g)	≥ 500	686	780	872

2.7 Enzyme pH stability

Amano's assessment of the stability (of enzyme activity) under varying pH conditions indicate that protein glutaminase is stable at the pH range of 5.0-7.0 as shown above in Figure 1.

2.8 Enzyme Thermal stability

Amano's assessment of the stability of enzyme activity under varying temperatures indicate that protein glutaminase is stable at a temperature range of 50-60°C. Figure 4 shows the residual activity when the enzyme is incubated for 10 or 60 minutes at various temperatures.

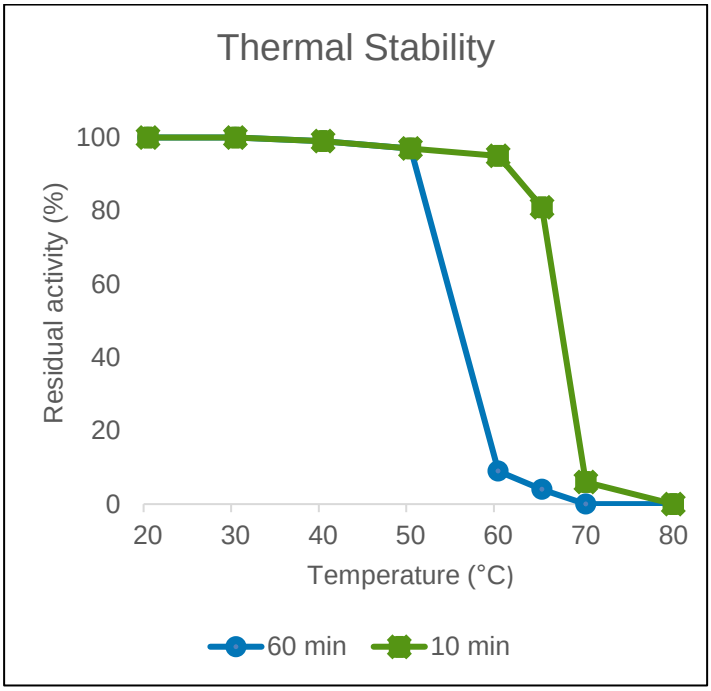

***Figure 4**. Stability of protein glutaminase activity when pre-incubated*

2.9 Long term stability

The stability of protein glutaminase activity was assayed over a 12-month period. Samples were sealed in airtight containers and stored at temperatures of -20°C, 4°C and 25°C (Figure 5). Results indicate that protein glutaminase activity is stable for at least 12 months from the date of manufacture under recommended storage conditions.

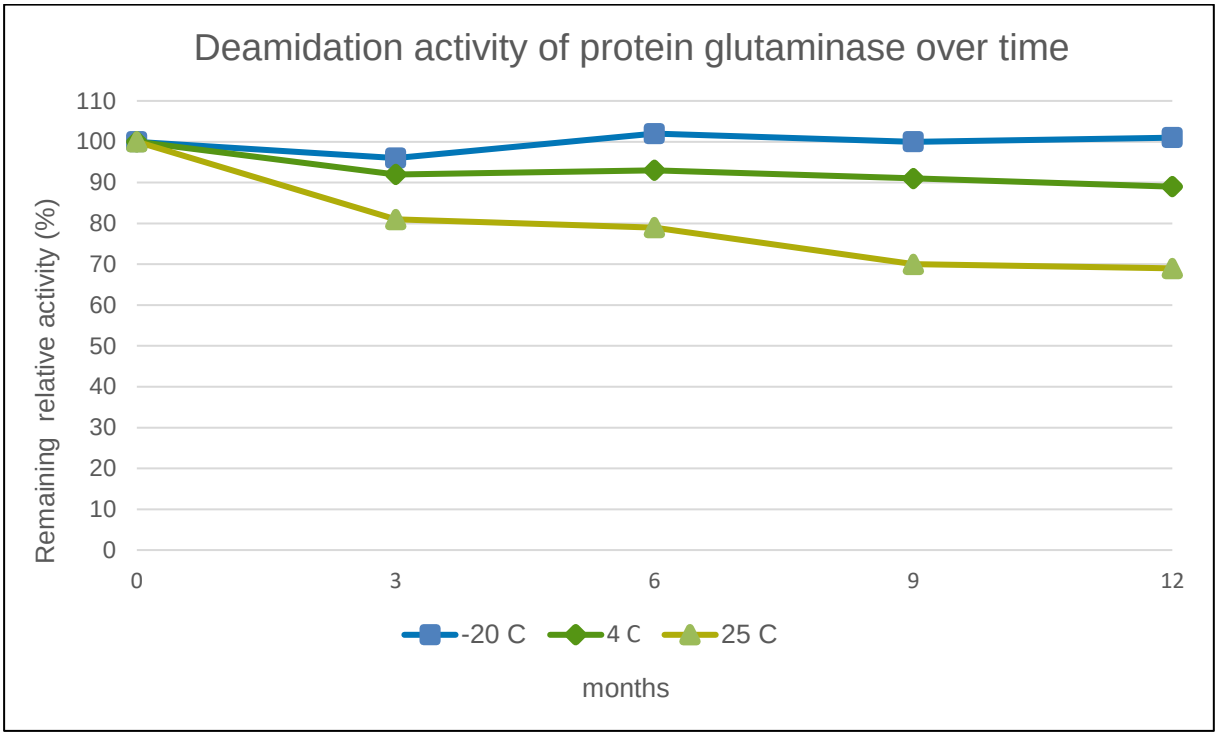

***Figure 5**. Long-term stability of protein glutaminase*

2.10 Food technology conclusion

FSANZ concludes that the use of protein glutaminase is effective in providing improvements in the solubility of proteins. This solubility promotes emulsification, foam stabilisation and gelling in various food processing applications. The food applications that would benefit from the use of protein glutaminase are baking, pasta/noodle making, the processing of milk and dairy, meat, fish, grains, yeasts products and egg based products. The evidence that Amano presented to support the proposed uses of the enzyme preparation provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and achieves its stated purpose. FSANZ also concludes that the enzyme performs its technological purpose during processing and manufacture of food after which it is inactivated therefore not performing any technological function in the final food. It is appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications for enzymes used in the production of food.

3 Hazard assessment

3.1 Background

The aims of the current hazard assessment were to:

- review the available data on the toxicology of protein glutaminase from *C. proteolyticum* to determine its safety as a food processing aid
- if appropriate, establish a health-based guidance value.

3.2 Hazard of the production organism

Two studies were submitted by the applicant, a pathogenicity and toxicity study using the parent strain *C. proteolyticum* 9670 and a second similar study using the production strain *C. proteolyticum* AE-PG.

Studies on the non-pathogenicity of Chryseobacterium proteolyticum strain 9670 and on the safety of the enzyme: protein-glutaminase [Scheuplein RJ et al 2007 Regulatory Toxicology and Pharmacology 49(2):79-89.]

The pathogenicity potential of *C. proteolyticum* strain 9670 was estimated according to the procedure of the National Research Council of Agriculture, Forestry and Fisheries, Japan (1985). This procedure uses cell viability in brain, kidney and liver tissue in combination with histological changes in the same tissues to estimate pathogenicity by intravenous and oral inoculation.

In the intravenous inoculation study, five-week old S1c:ICR male mice were inoculated by intravenous injection with *C. proteolyticum* at a dose of either 2.9 $x10^7$ or 2.9$x10^8$ cfu/mouse. Inoculation with the supernatant of the *C. proteolyticum* cell culture and the supernatant of the *C. proteolyticum* cell lysate were included to determine if exotoxins and endotoxins are produced by *C. proteolyticum*. The negative control was saline and the positive control was *Pseudomonas aeruginosa* IFO3919 at doses of 1.1$x10^6$ or 1.1$x10^7$ cfu/mouse. Ten mice were inoculated for all challenge and positive control groups and five mice were inoculated in the saline negative control group. In the oral inoculation study, five-week old S1c:ICR male mice were inoculated with *C. proteolyticum* at a dose of 1.3$x10^9$ cfu/mouse. The negative control was saline and no positive control was included in the oral inoculation study. Ten mice were included in the challenge group and five mice in the saline negative control group. For both the intravenous and oral inoculation studies, mice were observed daily for 14 days and weights measured on days 0, 1, 3, 7, 10 and 14. On day 14, the mice were killed, the brain, liver, lungs, spleen and kidneys were aseptically removed, and the tissues prepared for cell isolation and histopathology.

In both the intravenous and oral inoculation studies, no deaths were observed in any of the controls or *C. proteolyticum* and supernatant challenge groups. In the high *C. proteolyticum* dose group and cell lysate control, a transient decrease in locomotor activity, prone position and piloerection were observed 15 minutes after the inoculation, which disappeared after approximately 3 hours. No deaths or adverse effects were observed after oral inoculation with *C. proteolyticum* cells. No effects on body weight were observed in the *C. proteolyticum* challenge groups. Animals challenged with *P. aeruginosa* experienced dose related decreases in body weight, mortality and continuous piloerection and torticollis. Six mice in the *P. aeruginosa* high dose group died.

In both the intravenous and oral inoculation studies, mice challenged with *C. proteolyticum* or supernatants showed no signs of inflammation or infection in the observed organs and no viable cells were detected. In the *P. aeruginosa* low dose challenge group, purulent inflammation and viable cells were detected in 1 of 10 mice and in all four of the surviving

mice in the high dose group. The histopathological findings of the intravenous and oral challenge studies for *C. proteolyticum* and supernatants showed no gross abnormalities in the brain, lungs, liver, spleen or kidneys. Focal necrosis was found in the liver of 4 of 10 mice in the high dose *C. proteolyticum* intravenous challenge group and 1 of 10 mice in the *C. proteolyticum* cell lysate intravenous group. In the oral inoculation study, focal necrosis was observed in the liver of 1 of 5 animals of the saline control group. Extensive histopathological findings were observed in all mice challenged intravenously with a high dose of *P. aeruginosa*.

In supernatants of both the culture broth and cell lysate of the *C. proteolyticum* cells, 0.03 EU/ml of endotoxin was detected in the 100-fold diluted supernatant but not in the 1000-fold-diluent. This implies an endotoxin concentration between 3 and 30 EU/ml in the original preparations. This level of endotoxin is reported to be typical of the endotoxin levels found in drinking water and teas (1.0–533 EU/ml; reviewed by Petsch and Anspach 2000).

Based on these results, it is unlikely for *C. proteolyticum* strain 9670 to be pathogenic or toxigenic.

Single intravenous inoculation of PG-producing bacterial suspensions and single oral inoculation of PG-producing bacterial culture [Nagese (Study Director); Hashima Laboratory, Nihon Bioresearch Inc. Study Number 401635 (2016).]

Intravenous and oral inoculation studies were conducted using the production strain *C. proteolyticum* AE-PG to assess pathogenicity.

In the intravenous inoculation study, five-week old S1c:ICR male and female mice were inoculated by intravenous injection with *C. proteolyticum* at a dose of either 2.4 $x10^7$, $2.4x10^8$ or $2.4x10^9$ cfu/mouse. The negative control was saline. Ten mice, five male and five female, were inoculated with a *C. proteolyticum* suspension at the three different doses for the challenge groups or saline for the negative control group. In the oral inoculation study, five-week old S1c:ICR male mice were inoculated with *C. proteolyticum* at a dose of $2.1x10^9$ cfu/mouse. The negative control was saline. Ten mice, five male and five female, were included in the challenge group and the saline negative control groups. For both the intravenous and oral inoculation studies, mice were observed daily for 14 days and weights measured on days 0, 1, 3, 7, 10 and 14. On day 14, the mice were euthanised, the brain, liver, lungs, spleen and kidneys were aseptically removed, and the tissues prepared for cell isolation and histopathology.

In both the intravenous and oral inoculation studies, no deaths or abnormal findings were observed in any of the control mice or *C. proteolyticum* challenge groups. No effects on body weight were observed in the *C. proteolyticum* challenge groups.

In both the intravenous and oral inoculation studies, mice challenged with *C. proteolyticum* or supernatants showed no signs of inflammation or infection in the observed organs and no viable cells were detected. The histopathological findings of the intravenous and oral challenge studies for *C. proteolyticum* showed no gross abnormalities in the brain, lungs, liver, spleen or kidneys. Focal necrosis was found in the liver of 1 of 10 mice in the high dose *C. proteolyticum* intravenous challenge group. One of 10 mice challenged orally with *C. proteolyticum* and 1 of 10 mice in the oral saline negative control group developed focal necrosis in the liver.

Based on these results, it is unlikely for the enzyme production strain, *C. proteolyticum* strain AE-PG, to be pathogenic or toxigenic.

3.3 Hazard of the enzyme

Use of the enzyme as a food processing aid in other countries
Protein glutaminase from *C. proteolyticum* is approved for use in France. A generally recognised as safe (GRAS) notification for use of the enzyme as a food processing aid was submitted to the US FDA in 2008, and the US FDA responded that it had no questions on this submission in 2009.

Evaluation of toxicity studies with the enzyme
The test article in the submitted toxicity studies is stated to be protein glutaminase produced by *C. proteolyticum*. Although not specifically indicated in the reports, the Applicant has stated that the enzyme was produced by the production strain of *C. proteolyticum* (strain AE-PG), rather than by the parent strain.

Genotoxicity
Two genotoxicity studies were submitted by the applicant, a bacterial reverse mutation assay and a chromosomal aberration assay in mammalian cells.

Safety studies of protein-glutaminase produced by Chryseobacterium proteolyticum - reverse mutation test in bacteria. [Sui (Study Director); Hatano Research Institute, Food and Drug Safety Center study number M-03-067 (2004).]

The study was conducted in compliance with OECD Principles of Good Laboratory Practice (GLP) and in accordance with OECD Test Guideline (TG) 471, the Bacterial Reverse Mutation Test (July 1997). Relevant Japanese standards were also followed. The test item was protein glutaminase from *C. proteolyticum* strain AE-PG.

The tester strains used in the study were *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 and *Escherichia coli* WP2 uvrA. Based on the results of a dose range finding study, test doses of 313, 625, 1250, 2500 and 5000 µg protein glutaminase/plate were used in the mutagenicity tests. The test was conducted by the preincubation method with and without metabolic activation. Criteria for a positive response were a two-fold increase in the mean number of revertants compared with the negative control, and demonstration of a dose-response.

Growth inhibition by the test article was not observed in any of the tester strains at any dose, with or without S9. No precipitation of the test article was observed. There was no test article-induced increase in the number of revertant colonies compared with negative controls in any strain, with or without metabolic activation. In contrast, all positive controls produced the expected mutagenic response. Negative and positive control values were within the range of the historical control values for the test facility.

Protein glutaminase from *C. proteolyticum* strain AE-PG had no mutagenic activity under the conditions of this study.

Safety studies of protein-glutaminase produced by Chryseobacterium proteolyticum – chromosomal aberration test using Chinese hamster lung (CHK/IU) cells [Yamakage (Study Director); Hatano Research Institute, Food and Drug Safety Center study number G-03-046 (2004).]

The study was conducted in compliance with OECD Principles of GLP and in accordance with OECD TG 473, the *In Vitro* Mammalian Chromosomal Aberration Test (July 1997). Relevant Japanese standards were also followed.

Protein glutaminase produced by *C. proteolyticum* strain AE-PG was examined for its

potential to induce structural chromosome aberrations in both the absence and presence of S9 fraction, using Chinese hamster lung (CHL/IU) cells. The solvent and negative control was Na, K-Phosphate buffer (20 mmol/L, pH 70.). The positive control substances were mitomycin C in the absence of S9 fraction and cyclophosphamide in the presence of S9. Both a short-term treatment assay and a continuous exposure assay were conducted. The continuous exposure assay was conducted only in the absence of metabolic activation. The exposure period in the short-term treatment assay was 6 hours, and cells were harvested 18 hours following removal of the test article. The continuous exposure period was 24 hours. Colcemid® was added approximately 2 hours before the end of the culture period. All assays were conducted in duplicate.

Based on the results of a growth inhibition test 10 dose levels were selected for each of the cytogenetic tests. From these, three doses were selected for chromosome analysis based on an assessment of cell growth inhibition and mitotic index. These were 213, 425 and 675 µg/ml for the short-term treatment without S9; 106, 213 and 425 µg/mL for the short-term treatment with S9; and 63, 125 and 250 µg/mL for the continuous treatment. Clastogenicity was judged to be positive or negative based on statistical and biological significance.

No statistically significant increases in the number of cells with structural chromosomal aberrations or polyploid cells were observed at any dose of protein glutaminase in the short-term treatment groups with or without metabolic activation, or in the 24-hour continuous treatment group. The positive controls significantly induced structural chromosomal aberrations, confirming the validity of the test system for detection of clastogens.

Protein glutaminase from *C. proteolyticum* strain AE-PG did not induce structural chromosome aberrations or polyploid cells under the conditions of this study.

3.4 Animal studies

A 13-week oral toxicity study

13-week oral toxicity study of protein-glutaminase in rats [Yamaguchi (Study Director); Bozo Research Center study number B-5339 (2005)]

The study was conducted in compliance with relevant Japanese guidelines on GLP, toxicity studies and animal welfare. The test item was protein glutaminase from *C. proteolyticum* strain AE-PG with an enzyme activity of 549 U/ml. The test vehicle and negative control was water. Protein glutaminase was administered by oral gavage to Sprague-Dawley rats (Crj:CD(SD)IGS, 6 weeks of age, 12/sex/group) daily for 13 weeks. Rats were acclimated to the test facility environment for 7 days prior to the start of treatment, and animals were housed individually under standard laboratory conditions with free access to food and water.

Dose levels were selected based on results of a previous 2-week oral toxicity study of protein glutaminase in rats. Doses used in the main study were 63.5, 1269 and 2538 mg/kg bw/day, administered in dose volumes of 10 mL/kg bw.

Survival, clinical signs, body weight and food consumption were assessed throughout the course of the study, and an ophthalmological examination was performed before the start of the administration period and again in Week 13 of administration. Urine samples were collected in Weeks 5 and 13 and blood samples collected at scheduled necropsy. Animals were killed by exsanguination under anaesthesia on the day after the last administration of the test substance. Gross necropsy was conducted on all rats, organ weights were determined and tissues were preserved and processed for histopathological examination.

There were no mortalities over the course of the study and no clinical signs of toxicity were

observed. Body weight, body weight gain and food consumption in the treated groups were similar to controls. No treatment-related adverse effects were observed in ophthalmological examination, organ weight, urinalysis, blood chemistry, necropsy and histopathological examination. Haematological examination found a statistically significant prolongation of prothrombin time in high dose females, and significant reductions in fibrinogen in animals of both sexes in the high dose group compared with controls. However, these findings were not considered to be adverse in the absence of other accompanying changes in haematology parameters or histopathological findings.

The no observed adverse effect level (NOAEL) in this study was 2538 mg/kg bw/day, the highest dose tested. This equates to a total organic solids (TOS) content of 92.8 mg TOS/kg bw/day, based on a TOS content of the test substance of 0.928%, and administration of 10 mL/kg bw/day of the undiluted test substance at the highest dose.

3.5 Bioinformatic analysis for potential allergenicity

An *in silico* analysis was used to compare the amino acid sequence of protein glutaminase from *C. proteolyticum* strain AE-PG with that of known allergens using the Allergen Database for Food Safety. Searches were conducted to investigate whether there are matches for:

- more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty, or
- identity of 8 contiguous amino acids

These homology assessments are consistent with the recommendations of international organisations for screening of new food enzymes or newly expressed proteins in genetically modified plants for potential allergenicity (Codex 2003, 2009; WHO 2016).

None of these searches identified any matches with known allergens.

There is also no evidence of allergenicity to protein glutaminase from *C. proteolyticum* in countries with existing approvals for the enzyme (France and USA).

Overall, it is concluded that protein glutaminase from *C. proteolyticum* strain AE-PG does not have the characteristics of a potential food allergen and ingestion of any residual protein glutaminase in food products is unlikely to pose an allergenicity concern.

3.6 In vitro digestibility study

A digestion test using simulated gastric fluid and simulated intestinal fluid was provided by the applicant (Amano 2009). This study is not required, as the amino acid sequence of the enzyme has no similarity to any known allergens. However, it is noted that this study demonstrated that protein glutaminase from *C. proteolyticum* (strain unspecified) was rapidly digested in simulated pepsin-containing gastric fluids of pH 1.2 and pH 2.0, with no protein detected after incubation for 30 seconds or 2 minutes, respectively. No peptide fragments were detected at either pH. Protein glutaminase was also digested in simulated pancreatin-containing intestinal fluid (pH 6.8), although peptide fragments persisted up to a reaction time of 60 minutes, the longest reaction time tested.

3.7 Residual allergens from the culture medium

Soybean derivatives are used in the fermentation media and soy is a food allergen. However, analysis of protein glutaminase indicates that soybean protein is not detected above the limit of detection of 1 mg/kg. The protein glutaminase enzyme powder is further diluted with Cassava dextrin. Exposure to any potential residual soy allergens in final food products is therefore expected to be negligible and is not likely to be of allergenic concern.

3.8 Enzyme and source microorganism nomenclature

The International Union of Biochemistry and Molecular Biology (IUBMB), the internationally recognised authority for enzyme nomenclature, uses the name “protein-glutamine glutaminase” for enzymes with an EC number of 3.5.1.44 (IUBMB 2017).

C. proteolyticum is not currently listed in Schedule 18 of the Code. The species *C. proteolyticum* is not an approved bacterial name with standing in nomenclature, although Yamaguchi and Yokoe (2000) proposed that a new species with the name *C. proteolyticum* sp. nov. strain 9670 be designated as the type strain. The study by Yamaguchi and Yokoe (2000) described two strains (9670 and 9671), isolated from soil in Japan, for which DNA–DNA relatedness data and 16S rRNA sequence analysis indicated that the strains belong to the genus *Chryseobacterium*, but not to an already described species. The type strain, 9670, has been deposited in the Patent Microorganism Depository, National Institute of Bioscience and Human Technology (Tsukuba, Japan), as strain FERM P-17664 (Yamaguchi S, Yokoe, M 2000).

The Application included safety data for *C. proteolyticum* strain 9670 and the production strain AE-PG, which was derived from strain 9670 through chemical mutagenesis. While strain 9670 and AE-PG present no safety issues, there is currently insufficient evidence to assess the safety of *C. proteolyticum* at the species level as it is a novel bacterium with no other strains characterised or described in the scientific literature. This is consistent with the determination of the EFSA Panel on Biological Hazards, which recently recommended that *C. proteolyticum* was not suitable for inclusion on the list of microorganisms with a Qualified Presumption of Safety due to an insufficient body of evidence as all available safety studies refer to a single strain (EFSA 2016). Inclusion of the source microorganism in Schedule 18 should therefore refer to the species and strain name *Chryseobacterium proteolyticum* strain AE-PG.

3.9 Hazard assessment conclusion

There are no public health and safety concerns associated with the use of protein glutaminase from *C. proteolyticum* strain AE-PG as a food processing aid, based on the following considerations:

- *C. proteolyticum* strain AE-PG is not pathogenic or toxigenic
- Protein glutaminase from *C. proteolyticum* strain AE-PG was not genotoxic *in vitro*
- The no observed adverse effect level (NOAEL) in a 13-week repeated dose oral toxicity study in rats was the highest dose tested and corresponds to 2538 mg/kg bw/day or 92.8 mg TOS/kg bw/day. This is more than 200-fold higher than the Applicant’s estimate of an individual’s theoretical maximal daily intake (0.38 mg TOS/kg bw/day) based on the proposed uses, as stated in the Application
- Protein glutaminase from *C. proteolyticum* strain AE-PG does not have the characteristics of a potential food allergen and ingestion of any residual protein glutaminase in food products is unlikely to pose an allergenicity concern.

Based on the reviewed toxicological data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate. A dietary exposure assessment was therefore not required.

It is concluded that there are no safety concerns associated with the use of this enzyme as a food processing aid.

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