Petition to Amend Standard 1.3.3 of the Australia New Zealand Food Standards Code to Include Glutaminase from *Bacillus amyloliquefaciens* as a Processing Aid

Prepared for:

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Submitted to:

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LIST OF ABBREVIATIONS

AFSSA	l'Agence Française de Sécurité Sanitaire des Aliments
Amano Enzyme	Amano Enzyme Inc.
AMFEP	Association of Manufacturers of Fermentation Enzyme Products
EDI	estimated daily intake
FDA	U.S. Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
GLP	Good Laboratories Practices
GRAS	Generally Recognized as Safe
GTU	glutaminase units
HAP	hydrolysed animal protein
HVP	hydrolysed vegetable protein
NaCl	sodium chloride
NNS	Australian National Nutrition Survey
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
QA	Quality Assurance
TNO	TNO Nutrition and Food Sciences Institute
TOS	Total Organic Solids
U.S. EPA	U.S. Environmental Protection Agency
U.S.	United States

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GENERAL REQUIREMENTS

1.0 APPLICANT DETAILS

On behalf of Amano Enzyme Inc. (Amano Enzyme), Intertek Scientific and Regulatory Consultancy is submitting an application to amend Standard 1.3.3 of the Australia New Zealand Food Standards Code in order to include a new enzymatic processing aid of microbial origin. The processing aid is produced by Amano Enzyme Inc.

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Details of Other Parties Involved with the Application

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2.0 PURPOSE OF THE APPLICATION

Amano Enzyme Inc. (Amano Enzyme) wish to request an amendment to Standard 1.3.3 of the Food Standards Code to include glutaminase derived from *Bacillus amyloliquefaciens* as a processing aid intended for use in the manufacture of certain seasoning ingredients (*e.g.*, yeast extract, hydrolysed vegetable protein, and hydrolysed animal protein), as well as the manufacture of food products that are used as seasonings (*e.g.*, soy sauce, miso, vinegar, fish sauce). The trade name for the glutaminase enzyme preparation described in the current application is Glutaminase SD-C100S. This product comprises glutaminase concentrate diluted with sodium chloride (NaCl) to produce enzyme preparations containing 9% glutaminase concentrate.

Glutaminase is an enzyme that catalyses the conversion of L-glutamine to glutamic acid. The glutamic acid produced by this reaction is then converted to glutamate which is an important component of the quality and taste of the foods in which glutaminase is added. The use of glutaminase derived from *B. amyloliquefaciens* reduces the need for the addition of external sources of glutamate, such as monosodium glutamate, in these food products.

3.0 JUSTIFICATION FOR THE APPLICATION

3.1.1 Regulatory Impact Information

3.1.1.1 Cost and Benefit of the Proposed Change

The inclusion of glutaminase derived from *B. amyloliquefaciens* in the Australia New Zealand Food Standards Code as a processing aid will allow producers of certain seasoning ingredients (*e.g.*, yeast extract, hydrolysed vegetable proteins, and hydrolysed animal proteins) or foods used for seasoning (*e.g.,* soy sauce, miso, vinegar, fish sauce, *etc.*) to add a glutaminase step to their production process and avoid the addition of additives such as monosodium glutamate without affecting the quality or sensory characteristics of their food products. There will be no additional cost to the regulator if the processing aid is approved as the use of glutaminase derived from *B. amyloliquefaciens* will not impact the regulation of these food products. The use of glutaminase derived from *B. amyloliquefaciens* may affect the labelling of some food products in which it is intended for use as monosodium glutamate is required to be declared on the label of all foods products in Australia and New Zealand.

3.1.1.2 Impact on International Trade

The approval of glutaminase derived from *B. amyloliquefaciens* 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. Manufacturers and importers wishing to sell certain seasoning ingredients (*e.g.*, yeast extract, hydrolysed vegetable proteins, and hydrolysed animal proteins) and foods that are used as seasonings (*e.g.*, soy sauce, miso,

vinegar, fish sauce, *etc.*) that have been processed with the glutaminase enzyme in Australia/New Zealand would benefit.

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 glutaminase derived from *B. amyloliquefaciens* 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, 2013). The data pertaining to the glutaminase derived from *B. amyloliquefaciens* presented 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 glutaminase derived from *Bacillus amyloliquefaciens*, 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 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 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, 2013), 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 glutaminase derived from *Bacillus amyloliquefaciens* has a history of use in Europe and Japan. It is expected that the introduction of glutaminase derived from *Bacillus amyloliquefaciens* 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 Standard 1.3.3 of the Food Standards Code to include glutaminase derived from *Bacillus amyloliquefaciens* as a processing aid, to be the General Procedure (Subdivision D), Cost Category Level 2 (up to 650 hours). This is based

on the fact that FSANZ has approved processing aids derived from assessed and granted approval to other enzymes derived from the same organism, *Bacillus amyloliquefaciens,* and that Amano Enzyme's glutaminase product has already been approved and marketed in several other major jurisdictions (*e.g*., Europe, Japan) 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:

- Glutaminase SD-C100S received approval from l'Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for use as a processing enzyme in protein hydrolysates and yeast extracts (AFSSA, 2009).
- Glutaminase (microbial source not specified) is on the "List of Existing Food Additives" published by the Ministry of Health and Welfare of Japan (MHLW, 2014).

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.

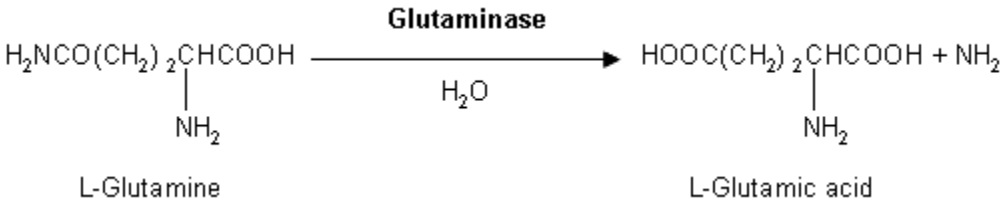
SECTION A: TECHNICAL DESCRIPTION OF GLUTAMINASE

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

Glutaminase is an enzyme catalysing the hydrolysis of L-glutamine to L-glutamic acid and ammonia. The enzyme breaks down L- and D-glutamine according to the reaction process illustrated in Figure A.1-1.

Figure A.1-1 Reaction Process for the Enzymatic Hydrolysis of L-Glutamine by Glutaminase



Amano Enzyme has prepared a glutaminase concentrate that is derived from a bacterial source (*Bacillus amyloliquefaciens*) by means of a fermentation process. The glutaminase concentrate is diluted with sodium chloride (NaCl) in order to produce Glutaminase SD-C100S, the enzyme preparation intended for use as a processing aid in food. As noted, the Glutaminase SD-C100S enzyme preparation contains 9% of the glutaminase enzyme. A full description of the manufacturing procedures for all commercial preparations is provided in Section A.5.

Based on the foregoing description, glutaminase derived from *B. amyloliquefaciens* would fall under the following classification within Standard 1.3.3 (Processing Aids):

(17) Permitted enzymes of microbial origin

The maximum proposed level of use for Glutaminase SD-C100S in the preparation of foods is 0.2%. Based on the dilution of the glutamine concentrate with NaCl, this level of use would be equivalent to 0.018% of the glutaminase concentrate in the final food product.

A.2 Information on the Identity of the Processing Aid

Systematic name:	L-glutamine aminohydrolase
Common name:	Glutaminase
IUBMB Enzyme Nomenclature of the active substance:	EC 3.5.1.2
CAS registration number of the active substance:	9001-47-2

The glutaminase preparation is produced by *B. amyloliquefaciens* strain GT2. Strain GT2 is not genetically modified organism but a chemically mutated production strain derived from strain NP. *B. amyloliquefaciens* has been used for many years for food or feedstuffs purposes or in the production of enzymes processing aids in Australia, New Zealand, Japan, the United States (U.S.), and Europe.

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 Glutaminase Activity

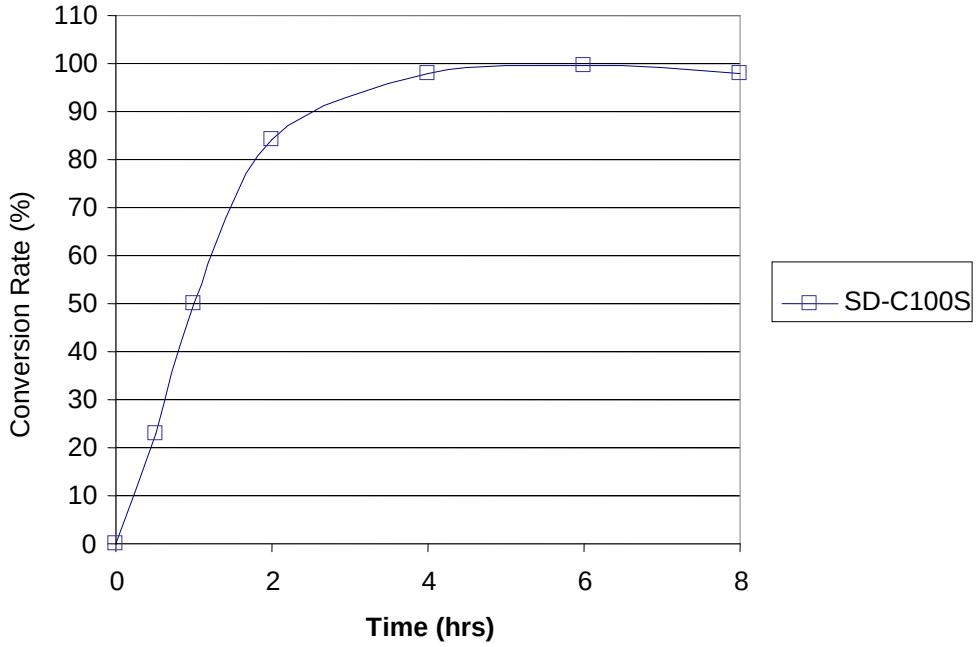
An analytical method for the detection and quantification of glutaminase activity is presented in Appendix A. In brief, this method measures the production of glutamic acid by a glutaminase preparation and allows the activity of the enzyme to be quantified. The glutaminase preparation (pH 6.0) is placed in a water bath and heated to 37°C prior to the addition of L-glutamine. After L-glutamine is added the reaction is allowed to run for exactly 10 minutes before being stopped by the addition of perchloric acid. An enzymatic determination of the glutamic acid content of the sample is conducted and the absorbance of the sample is measured to indicate activity. The results are reported in glutaminase units (GTU) per gram.

A.3.1.2 Characterization of Glutaminase Activity

The technical function of glutaminase is the conversion of L-glutamine to L-glutamic acid. The properties of the commercial glutaminase preparations have been examined in several assays conducted by Amano Enzyme. The results of the assays conducted to characterise the properties of the enzyme are presented in Appendix A and discussed in brief below. First the conversion rate of the glutaminase was examined for SD-C100S. SD-C100S was dissolved in a diluent comprising 100 mM acetate buffer and 10% Triton-X solution. The pH of the glutaminase solution was maintained at 6.0 and the reaction vessel was placed in a water bath to maintain a temperature of 37.0°C. A 0.5% L-glutamine solution was added to the reaction mixture and the reaction was allowed to proceed for up to 8 hours. Colorimetric

analysis was employed to determine the concentration of glutamic acid in the sample over time and to calculate the conversion rate. After a 4-hour incubation, 100% of the L-glutamine present in the reaction vessel was converted into L-glutamic acid when incubated with the glutaminase concentrate (Figure A.3.1.2-1).

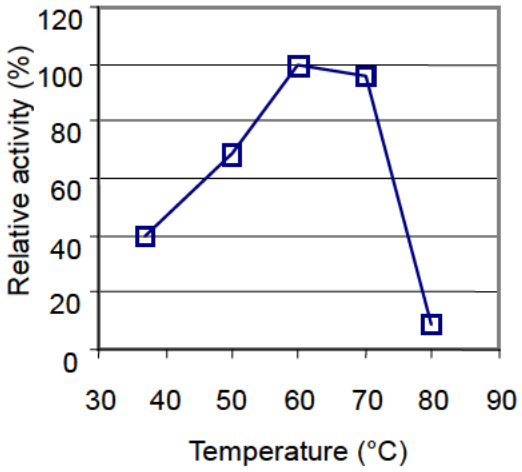
Figure A.3.1.2-1 Conversion rate of L-Glutamine into L-Glutamic Acid



Experimental conditions: pH6.0, 37°C, 0.5% L-Glutamine, 100mmol/L acetate buffer

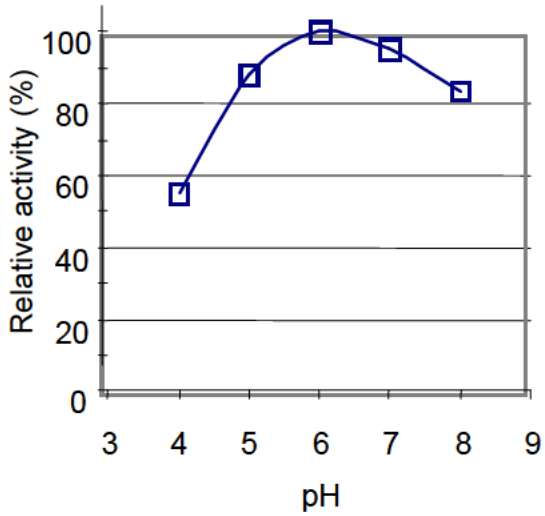
The effects of temperature and pH on the activity of the glutaminase concentrate were examined and the results are presented in Figures A.3.1.2-2 and A.3.1.2-3. In all assays the same experimental procedures described above were employed with the only modifications affecting the temperature of the water bath or the pH of the glutaminase solution. The effect of temperature and pH on the activity of SD-C100S 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. The activity of the sample at a given pH was compared to the activity measured when the reaction was run at a pH of 6.5. Based on the assays conducted, the peak activity of the glutaminase concentrate occurs at 65°C and a pH of approximately 6.

Figure A.3.1.2-2 Effect of the temperature on the activities of SD-C100S Glutaminase preparation



Experimental conditions: pH6.0, 5min., 1.0% L-Glutamine, 100 mmol/L acetate buffer, considering the activity at 37°C being 100%

Figure A.3.1.2-3 Effect of the pH on the activities of SD-C100S Glutaminase preparation



Experimental conditions: 37°C, 10 min.,1.0 % L-Glutamine, 50mmol/L Britton-Robinson buffer

A.3.1.3 Demonstration of Technological Function

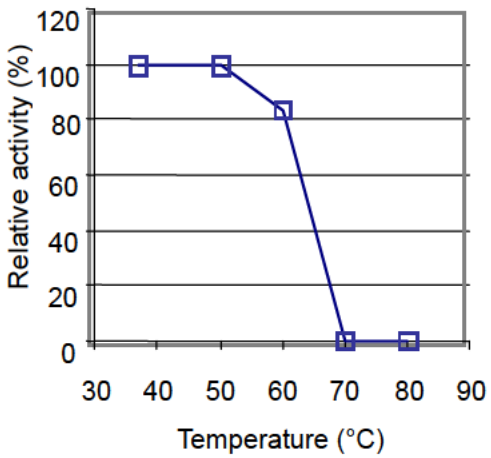
Glutaminase preparations have a history of use as processing aids in the manufacture of certain food ingredients such as miso and soy sauce, to increase the glutamate content of these foods (Harayama and Yasuhira, 1991; Nandakumar *et al.*, 2003). Therefore, the ability of glutaminase enzyme preparations to increase the levels of glutamate in foods has been well demonstrated. For example, Harayama and Yasuhira (1991) have shown that

addition of a glutaminase enzyme preparation at 0.05% (0.5 g enzyme preparation/kg food) during miso fermentation resulted in the conversion of L-glutamine to L-glutamate. Based on the experience in the food industry, and taking into account the enzyme activity of the Glutaminase SD-C100S preparation (see Section A.3.1.2), Amano Enzyme proposes the maximum use level for Glutaminase SD-C100S to be 0.2% when used during the manufacture of certain seasonings (*e.g.*, yeast extracts, hydrolysed vegetable proteins, hydrolysed animal protein, soy sauce, miso, vinegar, fish sauce, *etc.*) which equates to 0.018% of the glutaminase concentrate.

A.3.2 Stability

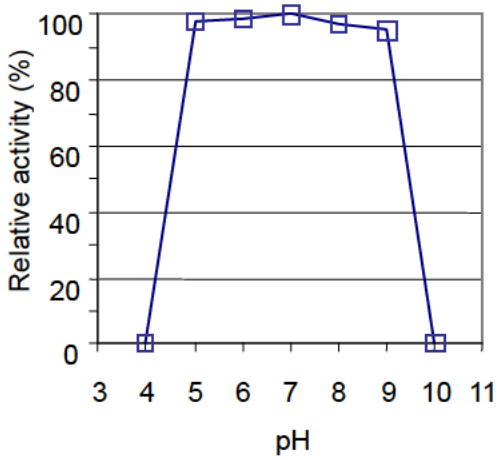
The stability of the final enzyme preparation, Glutaminase SD-C100S, has been assayed as a function of temperature, pH, concentration, and time. As the enzyme activity was considered the primary marker of the stability of the preparations, the experimental procedures described in Section A.3.1 were employed to assess the stability of Glutaminase SD-C100S. The only change to the experimental procedures was the duration of the incubation. In the assessments, the reaction was allowed to run for a period of 10 minutes before it was stopped and the production of glutamic acid was measured. The results of the assessment of the thermal and pH stability of Glutaminase SD-C100S are presented in Figures A.3.2-1 and A.3.1-2.

Figure A.3.2-1 Thermal stability of the activities of SD-C100S Glutaminase preparation



Experimental conditions: pH 6.0, 60 min., 50mmol/L acetate buffer, considering the activity at 37°C being 100%

Figure A.3.2-2 pH stability of the activities of SD-C100S Glutaminase preparation

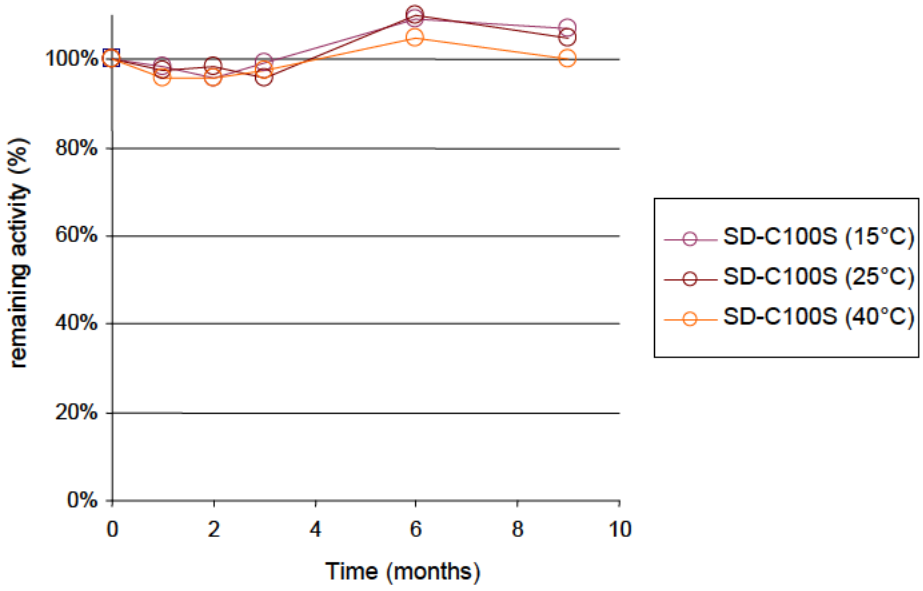


Experimental conditions: 37°C, 16 hrs, 50mmol/L Britton -Robinson buffer

The results of the assessment of stability under varying temperature and pH conditions indicate that Glutaminase SD-C100S is stable at up to 60°C and in a pH range of 5 to 9.

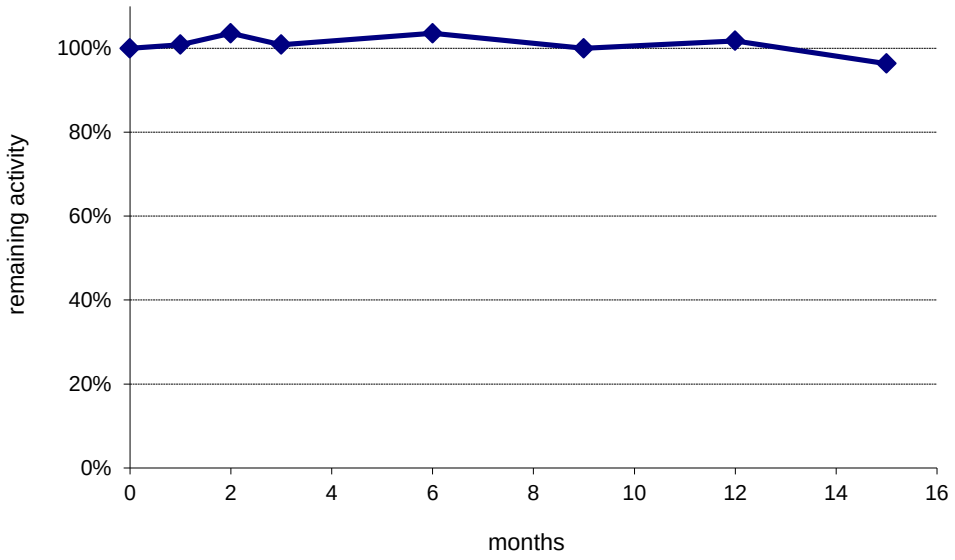
The results of the assessments of the stability over time for Glutaminase SD-C100S are presented in Figures A.3.2-3 and A.3.2-4. In the first assay, batches of Glutaminase SD-C100S were stored at 15°C, 25°C, and 40°C for a period of 9 months. Samples were collected from the batches at baseline and 1, 2, 3, 6, and 9 months of storage and the glutaminase activity of the batches were measured. The results indicate that the preparation is stable over 9 months at temperatures up to 40°C.

Figure A.3.2-3 Stability of Glutaminase SD-C100S Preparations Over 9 months



A second assessment of stability was conducted in which Glutaminase SD-C100S was stored according to the conditions recommended by the manufacturer. In this assessment batches of the finale enzyme preparation was placed into an airtight bag and kept at room temperature for a period of 15 months. Samples were collected at 0, 1, 2, 3, 6, 9, 12, and 15 months of storage. Stability was measured by the activity of the Glutaminase SD-C100S relative to the activity measured at baseline.

Figure A.3.2-4 Stability of Glutaminase SD-C100S Stored at Room Temperature for 15 months



The results of this stability assessment indicate that the Glutaminase SD-C100S is stable for at least 12 months when stored according to the recommended conditions.

A.3.3 Possible Interactions with Food Constituents

Glutaminase is an enzyme which acts on single substrate and would therefore, not be expected to act on other constituents in the food. The glutaminase preparation does contain additional enzymes secreted by *B. amyloliquefaciens* during fermentation, and the potential for secondary enzyme activity is discussed in Section A.3.4. The recommended conditions of use for SD-C100S developed by Amano Enzyme specify that when employed as a processing aid, the enzyme preparation must be inactivated either by temperature or pH changes. Amano Enzyme recommends that the inactivation be accomplished by changing the pH of the food so that it is lower than 5 or greater than 9 or by increasing the temperature above 60°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 glutamine present in non-target foods.

A.3.4 Characterisation of Secondary Activities

During the production process for glutaminase, the enzyme of interest is secreted by the bacteria during the fermentation process. The secondary activities identified during the production of glutamate rich yeast extract and hydrolysed vegetable or animal protein are those of the protease, α-amylase, and peptidase present in the final enzyme preparation. All three of these enzymes could react with reagents presented in the food; however, the production of all these foods involve the early hydrolysis of proteins in order to free the L-glutamine for reaction with Glutaminase SD-C100S. As a result, it is not anticipated that secondary activities would be extensive in the final food product.

Secondary activities have been determined in one lot. The results of the determinations are presented in Table A.3.4-1 and the assessments are included in Appendix A. The activity levels reported in Table A.3.4-2 take into account the dilution of the glutaminase concentrate with NaCl to prepare the final commercial enzyme preparation.

Table A.3.4-1 Glutaminase Activity and Secondary Enzymatic Activity in Glutaminase Concentrate Preparations

Enzyme	Glutaminase spray dried concentrate (Lot. GT0704132.00SP)
Glutaminase (GTU/g)	1970
Protease (PU/g)	145000
α-Amylase (AU/g)	136000
Peptidase(U/g)	< 0.25

Abbreviations: AU = Amylase units; GTU = glutaminase units; PU = protease units; U = peptidase units.

Table A.3.4-2 Glutaminase Activity and Secondary Enzymatic Activity in Commercialised Glutaminase Preparations

	Glutaminase SD-C100S (Lot. GT0704132.00SP)
Glutaminase (GTU/g)	110
Protease (PU/g)	8100[1]
α-Amylase (AU/g)	7600[1]
Peptidase (U/g)	< 0.014[1]

Abbreviations: AU = Amylase units; GTU = glutaminase units; PU = protease units; U = peptidase units.
[1] Calculation value based on the enzymatic activity of concentrate.

The results of the assessment indicate that the secondary activities of the protease, α-amylase, and peptidase are present in both the glutaminase concentrate and Glutaminase SD-C100S. As a result an assessment of secondary activity of all secondary enzymes was conducted within food matrices. In these assessments, mixtures of hydrolysed vegetable proteins and yeast extracts were incubated with 0.01 or 0.1% Glutaminase SD-C100S. This incubation period was up to 90 minutes in length and intended to mimic the real production process for these foods. At the end of the incubation period the remaining activity of all

enzymes were assessed. The results of these assessments are presented in Tables A.3.4-3 and A.3.4-4 and the assessment report is resented in Appendix A.

Table A.3.4-3 Residual Activities in Yeast Extracts Incubated with Glutaminase SD-C100S

Experimental Conditions		2% yeast extract + 0.01% glutaminase SD-C100S		
Temperature (°C)	Time (min)	Glutaminase (GTU/mL)	Protease (U/mL)	α-Amylase (U/mL)
Control	0	0.01000	0.810	0.7600
70	5	0.00581	0.066	0.0221
	10	0.00255	0.012	<LD
	20	0.00078	<LD	<LD
	30	0.00007	<LD	<LD
80	5	0.00014	<LD	<LD
	10	0.00070	<LD	<LD
	20	<LD	<LD	<LD
	30	<LD	<LD	<LD
90	5	<LD	<LD	<LD
	10	<LD	<LD	<LD
	20	<LD	<LD	<LD
	30	<LD	<LD	<LD

Abbreviations: GTU = glutaminase units; HVP = hydrolysed vegetable protein; LD = limit of detection; min = minutes; PU = protease units; U = peptidase units.
Detection limits = glutaminase: 0.00007 GTU/mL; α-amylase: 0.0007 U/mL; protease: 0.004 U/mL

Table A.3.4-4 Residual Activities in Hydrolysed Vegetable Proteins Incubated with Glutaminase SD-C100S

Experimental Conditions		10% HVP + 0.1% glutaminase SD-C100S		
Temperature (°C)	Time (min)	Glutaminase (GTU/mL)	Protease (U/mL)	α-Amylase (U/mL)
Control	0	0.10000	8.140	7.5900
70	5	0.01857	<LD	7.7000
	10	0.00362	<LD	4.3200
	20	0.00043	<LD	1.1500
	30	<LD	<LD	0.1790
80	5	0.00021	<LD	1.4400
	10	<LD	<LD	0.1460
	20	<LD	<LD	0.0050
	30	<LD	<LD	<LD
90	5	<LD	<LD	0.0137
	10	<LD	<LD	0.0060
	20	<LD	<LD	<LD
	30	<LD	<LD	<LD

Abbreviations: AU = amylase units; GTU = glutaminase units; HVP = hydrolysed vegetable protein; LD = limit of detection; min = minutes; U = peptidase units.
Detection limits = glutaminase: 0.00007 GTU/mL; α-amylase: 0.0007 U/mL; protease: 0.004 U/mL

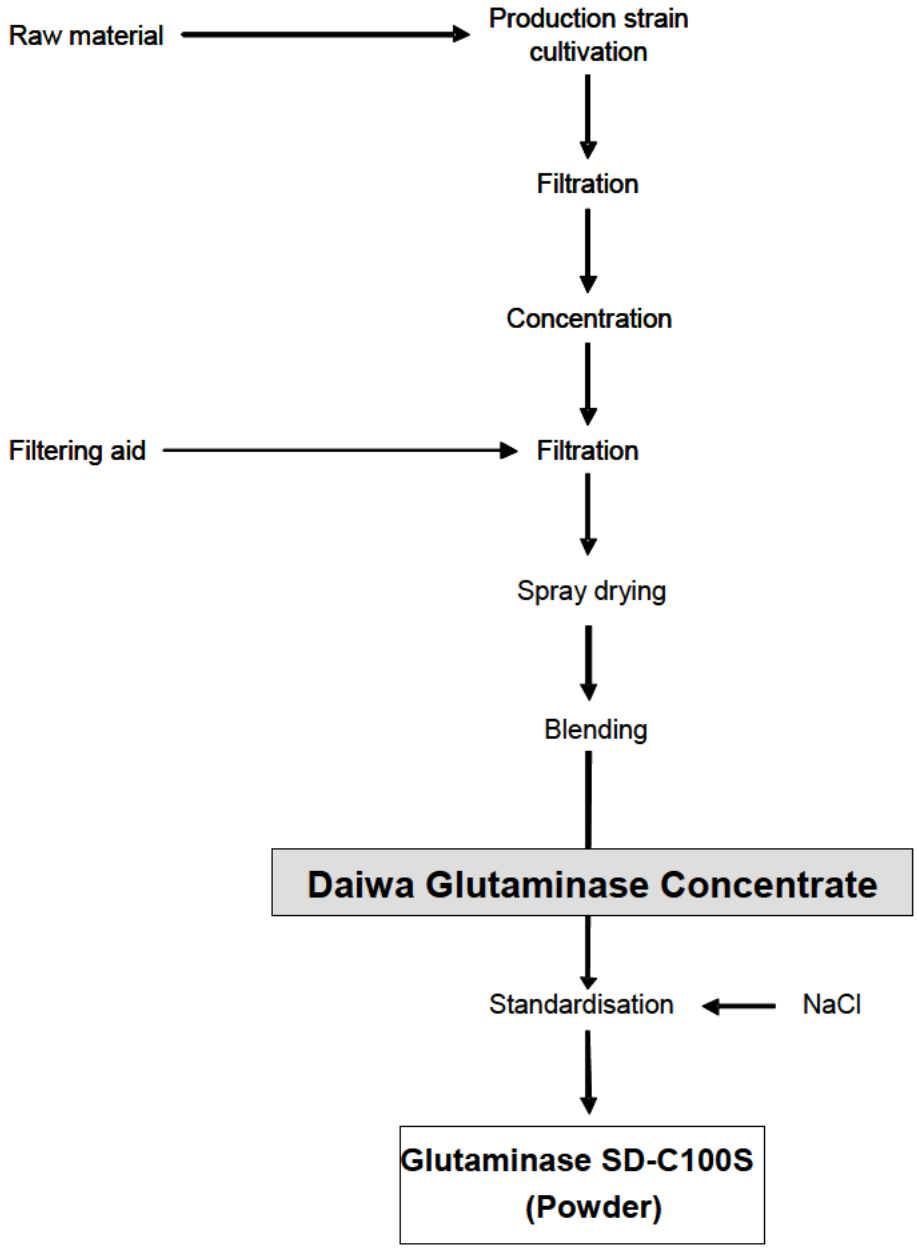
For incubations conducted at high temperatures (greater than 70°C), the activity of all enzymes present in the food drops below the limit of detection after 30 minutes with several dropping below the limit after only 5 minutes.

A.4 Manufacturing Process

A.4.1 Manufacturing Steps

A schematic overview of the overall manufacturing process for the Glutaminase SD-C100S product is provided in Figure A.4.1-1.

Figure A.4.1-1 Summary of the Manufacturing Process for Glutaminase SD-C100S



In brief, the production of the glutaminase concentrate begins with the fermentation of *B. amyloliquefaciens* under standard culturing conditions. Once the fermentation is complete, the cultures are filtered twice using high pressure to remove the culture media, before undergoing an ultrafiltration to concentrate the extracellular glutaminase, followed by one final sterile filtration to remove any remaining bacteria. The glutaminase concentrate is then spray dried and blended to produce a powdered glutaminase concentrate. To prepare the commercial enzyme preparation (Glutaminase SD-C100S), the powdered glutaminase

concentrate is diluted by the addition of NaCl. The Glutaminase SD-C100S preparation comprises 91% NaCl and 9% glutaminase concentrate.

A number of production controls are employed to maintain and monitor the purity of the microbial source and to avoid strain drift, as discussed in Section D.3. The enzyme preparation is produced according to the ISO 9001 quality control 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) certification and certificate of conformity to ISO 9001 are provided in Appendix A.

A.4.2 Raw Materials

The raw materials employed in the production of Glutaminase SD-C100S is listed in Table A.4.2-1 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 Glutaminase SD-C100S 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.4.2-1 Raw Materials and Processing aids Used in the Production of Glutaminase

Substance	Grade	Function	Status in Australia and New Zealand (FSANZ, 2014)
Yeast extract	Food	Nutrient	Food ingredient
Trehalose	Food	Nutrient	Approved for use as a novel food or food ingredient
Lactose	Food	Nutrient	Food ingredient
Defatted soybean	Food	Nutrient	Food ingredient
Corn steep liquor	Food additive	Nutrient	Food ingredient
Soybean oil	Food	Nutrient	Food ingredient
Ammonium sulphate $[(NH_4)_2SO_4]$	Food additive	Nutrient	Permitted for uses and a microbial nutrient (Standard 1.3.3)
Na_2CO_3	Food additive	Nutrient	Approved for use a food additive when used in accordance with Good Manufacturing Process (GMP) (Standard 1.3.1)
Water	Food ingredient	Nutrient	Not applicable
Dextrin	Food ingredient	Nutrient	Approved for use a food additive when used in accordance with GMP (Standard 1.3.1)
Calcium Chloride ($CaCl_2$)	Food additive	Processing aid	Approved for use a food additive when used in accordance with GMP (Standard 1.3.1)

Table A.4.2-1 Raw Materials and Processing aids Used in the Production of Glutaminase			
Substance	**Grade**	**Function**	**Status in Australia and New Zealand (FSANZ, 2014)**
Disodium phosphate (Na_2HP0_4)	Food additive	Processing aid	Approved for use a food additive when used in accordance with GMP (Standard 1.3.1)
Sodium Hydroxide (NaOH)	Food additive	Processing aid	Permitted for use in the production of processing aids (Standard 1.3.3)
Acetic acid ($C_2H_4O_2$)	Food additive	Processing aid	Approved for use a food additive when used in accordance with GMP (Standard 1.3.1)
Perlite	Filter aid	Processing aid	Permitted for use in the production of processing aids (Standard 1.3.3)
Diatomaceous earth	Filter aid	Processing aid	Permitted for use in the production of processing aids (Standard 1.3.3)
Sodium hypochlorite (NaClO)	Cleaning agent	Processing aid	Permitted in the manufacture of all foods as a bleaching, washing, or peeling agent at a maximum level of 1 m/kg

A.5 Specification for Identity and Purity

A.5.1 Product Specifications

The specifications for the glutaminase concentrate and the final SD-C100S enzyme preparation is presented in Table A.5.1-1.

Table A.5.1-1 Specification for Amano Enzyme Glutaminase Preparations			
Parameter	**Criteria**	**Product**	
		Glutaminase Concentrate	**Glutaminase SD-C100S**
Appearance	-	Light brown powder	Light brown powder
Glutaminase activity	not less than	1,800 GTU/g[1]	100-120 GTU/g[1]
Loss on drying (105°C)	not more than	8%	4%
Lead	not more than	2 µg/g	2 µg/g
Heavy metals (as Pb)	not more than	40 µg/g	40 µg/g
Arsenic (as As)	not more than	1 µg/g	1 µg/g
Cadmium	not more than	0.5 µg/g	0.5 µg/g
Mercury	not more than	0.5 µg/g	0.5 µg/g
Coliforms	not more than	30 CFU/g	30 CFU/g
Escherichia coli	negative in	2.22 g	2.22 g
Salmonella	negative in	25 g	25 g
Viable bacteria count	not more than	5 10^4 CFU/g	5 10^4 CFU/g

Abbreviations: CFU = colony forming units; GTU = glutaminase activity.
[1] One unit of glutaminase activity (GTU) is defined as the amount of enzyme that forms 1 µmol of L-glutamic acid per minute from L-glutamine in the reaction at 37°C, pH 6.0.

A specification for glutaminase enzyme has not been published by any authoritative sources. As a result, the specifications for the glutaminase concentrate, Glutaminase SD-C100S was compared to the specifications for heavy metals listed in Standard 1.3.4 of the Australia New Zealand Food Standards Code which addresses the purity and identify of food additives and processing aids. The specifications for the limits of the glutaminase concentrate and the final enzyme preparation meet the limits for lead, arsenic, cadmium, and mercury established in Standard 1.3.4.

A.5.2 Batch Analysis

Extensive analysis of production scale batches of the final SD-C100S preparation has been conducted to ensure that this preparation meets appropriate purity criteria, including the heavy metals specifications established in Standard 1.3.4 (FSANZ, 2014). The results of the assessments are provided in Table A.5.2-1 and in Appendix A. Additionally, analysis of 3 production batches of the glutaminase concentrate confirmed the absence of any antibiotic activity (see Appendix A).

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Table A.5.2-1 Analysis of Production Scale Batches of Glutaminase SD-C100S							
		Glutaminase SD-C100S					
Parameter	**Unit**	**P7EB161**	**P8AA791**	**P8AA891**	**P8ID065**	**P8ID066**	**P8HC161**
Heavy metals							
Arsenic	mg/kg	<0.10	<0.10	<0.10	nd	nd	nd
Cadmium	mg/kg	<0.01	<0.01	<0.01	nd	nd	nd
Mercury	mg/kg	<0.01	<0.01	<0.01	nd	nd	nd
Lead	mg/kg	<0.05	<0.05	<0.05	nd	nd	nd
Heavy metal (as Pb)	mg/kg	<1.00	<1.00	<1.00	nd	nd	nd
Microbiological purity							
Total viable count of aerobic mesophilic micro-organisms	CFU/g	<300	<300	<300	nd	nd	nd
Coliform bacteria	/g	Negative	negative	negative	nd	nd	nd
Escherichia coli	/2.22g	Negative	negative	negative	nd	nd	nd
Salmonella	/25g	Negative	negative	negative	nd	nd	nd
Clostridium perfringens	/0.1g	Negative	negative	negative	nd	nd	nd
Coagulase positive staphylococci	/g	Negative	negative	negative	nd	nd	nd
Campylobacter jejuni/coli	/10g	Negative	negative	negative	nd	nd	nd
Listeria Monocytogenes		Negative	negative	negative	nd	nd	nd
Clostridia	/g	Negative	negative	negative	nd	nd	nd
Antibacterial activity					negative	negative	negative
Mycotoxins							
Aflatoxin B1	µg/kg	nd	nd	nd	<0.5	<0.5	<0.5
Aflatoxin B2	µg/kg	nd	nd	nd	<0.5	<0.5	<0.5
Aflatoxin G1	µg/kg	nd	nd	nd	<0.5	<0.5	<0.5
Aflatoxin G2	µg/kg	nd	nd	nd	<0.5	<0.5	<0.5
Ochratoxin A	µg/kg	nd	nd	nd	<0.5	<0.5	<0.5

Table A.5.2-1 Analysis of Production Scale Batches of Glutaminase SD-C100S							
		Glutaminase SD-C100S					
Parameter	**Unit**	**P7EB161**	**P8AA791**	**P8AA891**	**P8ID065**	**P8ID066**	**P8HC161**
Sterigmatocystin	µg/kg	nd	nd	nd	<20	<20	<20
Zearalenone	µg/kg	nd	nd	nd	<50	<50	<50
T2 toxin	µg/kg	nd	nd	nd	<200	<200	<200

Abbreviations: CFU = colony forming units; nd = 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, 2013). Therefore, this section is not relevant to the use of glutaminase derived from *B. amyloliquefaciens*

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

Glutaminase concentrate has been used in Japan for many years in food processing, and it is currently on the "List of Existing Food Additives" published by the Ministry of Health and Welfare of Japan (MHLW, 2014). Glutaminase has been employed in the production of soy sauces since 1991 and in the production of miso since 1992 (Amano Enzyme, 2005). The use of glutaminase as a processing aid for the production of hydrolysed vegetable protein has been ongoing since 2003. Glutaminase enzymes derived from *B. amyloliquefaciens* have a long history of use in Japan as they were first reported in the public literature in 1988 (Shimizu *et al*., 1991).

Recently, Glutaminase SD-C100S received approval from l'Agence Française de Sécurité Sanitaire des Aliments (AFSSA) for use as a processing enzyme in protein hydrolysates and yeast extracts (AFSSA, 2009). The safety evaluation and conclusions of the AFSSA assessment regarding the use of Glutaminase SD-C100S as a processing aid are described in greater detail in Section C.4.

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

As mentioned in Section C.1, glutaminase enzyme preparations have a wide history of use in food processing. To further support the safety of Glutaminase SD-C100S, several toxicity studies have been conducted to assess the safety of the glutaminase concentrate (prior to dilution with NaCl). The potential mutagenic and genotoxic activity of the glutaminase concentrate preparation 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 safety of the NaCl component of Glutaminase SD-C100S is described in brief in Section C.2.2.

C.2.1 Glutaminase Concentrate

The full study reports of the unpublished toxicological assessments conducted with the glutaminase concentrate is provided in this application. It should be noted that in the reports prepared by the TNO Nutrition and Food Sciences Institute (TNO) the purity of the sample for lot GT70625L12 was reported to be 10.8% for the sample. In fact, this is not the purity but only the content of glutaminase proteins in the preparation as demonstrated in Table C.2.1-1.

Table C.2.1-1 Composition of Glutaminase Concentrate (Batch No. GT812185.00UP)				
Component		**Enzyme**	**Activity**	**Composition**
Moisture, g/100g	5.6	—	—	—
Protein, g/100g	58.2	Glutaminase protein	About 1,800 GTU/g	10.80%
		Amylase protein	av. 45,550 U/g	47.40%
		Protease protein	av. 6,596 U/g	
		Other protein	Unknown	
Ash, g/100g	11	—	—	—
Carbohydrate, g/100g	25.2	—	—	—

Abbreviation: GTU = glutaminase units.

C.2.1.1 Mutagenicity and Genotoxicity

Two assays were conducted to assess the potential mutagenicity and genotoxicity of the glutaminase concentrate including a reverse mutation assay and a chromosomal aberration assay (van Delft, 1998 [unpublished]; van Delft and de Vogel, 1998 [unpublished]). The assay was conducted in *Salmonella typhimurium* and *Escherichia coli* by the TNO Nutrition and Food Sciences Institute. The assay was carried out in accordance with the guidelines of the Organisation for Economic Co-operation and Development (OECD), the United States Environmental Protection Agency (U.S. EPA), the Japanese Ministry of Labour, Agriculture, Forestry and Fisheries and the Japanese Ministry of Health and Welfare. The methodology employed was in compliance with OECD guideline 471 on the assessment of genetic toxicity and the EEC protocol B.14 on mutagenicity.

Four *Salmonella typhimurium* strains (TA98, TA100, TA1535 and TA1537) and one *Escherichia coli* strain (WP2uvrA) were assayed with and without metabolic activation (S9 mix). The assay was conducted in compliance with Good Laboratories Practices (GLP) and internal TNO Quality Assurance (QA) operating procedure. The test substance was a concentrate of glutaminase applied to the strains at levels of 62, 185, 556, 1,667, and 5,000 µg/plate and was administered in the presence and absence of S9 mix. The recommended positive controls for each strain were assayed to ensure that the test was functioning properly. Three assays were performed in total with the first 2 assays performed according to the plate incorporation method and the third one performed according to the liquid-culture method (for substances containing proteins). Moreover, because the sample was not sterile, the last 2 assays were performed after sterilisation by filtration. All plates were incubated at 37°C for a period of 3 days and assessed in triplicate.

The results of this study indicate that glutaminase concentrate did not inhibit the growth of the bacteria at doses of up to 5,000 µg/plate. In the plate incorporation assays, a 2-fold or greater increase of revertant colonies was observed at the highest dose for strain TA1535 in the presence of the S9 mix. A slight dose-related increase of revertant colonies was also observed for strain TA100 in the presence of the S9 mix. The third confirmation assay using liquid-culture method failed to show any mutagenic effect. It was suggested by the authors

that these observations may have resulted from the fact that the test compound was not sterile and was observed to produce few colonies when plated on its own. The authors also noted that the protein content of the glutaminase concentrate may have acted as a source of L-histidine or its precursors and therefore, been the cause of a false positive reading in the plate incorporation assays. The negative results obtained in the liquid-culture method support this opinion.

As the effects noted in the assay were considered to be false-positive results on the basis of sterility and protein content, it was concluded that under the conditions employed in the performed bacteria reverse mutation test, glutaminase concentrate was not mutagenic in *S. typhimurium* strains TA1535, TA1537, TA98 and TA100 and in *E. coli* strain WP2uvrA, both in the presence or in the absence of metabolic activation.

It addition to the assay described above, a chromosomal aberration assay was conducted as an assessment of the genotoxicity of the glutaminase concentration (van Delft and de Vogel, 1998 [unpublished]). The mammalian cell chromosomal aberration study of the Enzyme RP-1 was carried out in 1998 by the TNO Nutrition and Food Sciences Institute. The assay was carried out in accordance with the guidelines of the OECD, the U.S. EPA, the Japanese Ministry of Labour, Agriculture, Forestry and Fisheries and the Japanese Ministry of Health and Welfare. The methodology employed was in compliance with OECD guideline 473 on the assessment of genetic toxicity and the EEC protocol B.10 on mutagenicity.

CHO K-1 cells from Chinese hamster ovaries were assayed in the presence and absence of metabolic activation (S9 mix). The assay was conducted in compliance with GLP and internal TNO QA operating procedures. The test substance was a concentrate of glutaminase derived from *B. amyloliquefaciens*. The tests included in this assay first examined the solubility and physical effects of doses of 625, 1,250, 2,500, and 5,000 µg/mL of the glutaminase concentrate. A preliminary assay was conducted in order to assess the cytotoxicity of the glutaminase preparation at doses of 50 to 5,000 µg/mL. The results of this test indicated that in the presence of S9 the glutaminase concentrate was cytotoxic at all doses and in the absence of S9 the glutaminase concentrate was cytotoxic at doses greater than 75 µg/mL. As a result the doses selected for the chromosomal aberration assay ranged between 0.05 and 30 µg/mL in the presence of the S9 mix and ranged between 0.5 and 200 µg/mL in the absence of the S9 mix. Cyclophosphamide was employed as a positive control for the assays conducted in the presence of metabolic activation and mitomycin was employed as a positive control for those conducted without metabolic activation. In all assays the CHO K-1 cells were treated for a period of 4 hours and harvested for examination after 18 hours.

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

C.2.1.2 Repeat Dose Toxicity Assay

A 13-week subchronic oral toxicity study (dietary administration) was performed by the TNO Nutrition and Food Sciences Institute (Appel, 1999). The study was performed according to OECD guidelines for the testing of chemicals 408, with the EEC guidelines and the U.S. Food and Drug Administration (FDA) guidelines for the testing of food additives. The study was conducted in compliance with GLP and internal TNO QA operating procedures. Young Wistar outbred (Cr1:(WI)WU BR) rats 4 weeks of age, weighing between 126.8 and 158.3 g at the start of the experimental period, were obtained from Charles Rivers (Sulzfeld, Germany). The rats intended for use in the dose range-finding study were housed in quarantine for a 5-day period while the rats intended for use in the 13-week study were allowed to acclimate for 11 days. During both periods the health of rats was examined and monitored. All animals were housed in groups of 5 and the cages were kept in a room with 10 air changes per hour, a temperature of 22 ± 3°C, and relative humidity of 30 to 70%. The lighting of the room was artificial and set to a 12-hour light-dark cycle. In the final week of the experimental period, 10 rats per sex were selected from each dose group and housed singly in metabolic cages for 16 hours.

Feed and drinking water were provided *ad libitum* through the acclimation and experimental periods. The administered diets contained 0 (control), 0.2, 0.6, or 2.0% glutaminase concentrate with each dose provided to 20 rats per sex. The test article was mixed with a powered basal diet to reach the prescribed concentrations and the stability, homogeneity and concentration of the test article in the diet were monitored. The rats were weighed on the first day of the experimental period, once weekly thereafter, and on the final day of the experimental period prior to autopsy. Clinical examinations of the health of the animals were conducted daily. Food consumption was measured weekly and water intake was measured on 4 consecutive days during weeks 1, 6, and 12 of the experimental period. At the end of the experimental period blood samples were collected from 10 male and female rats per dose for haematological and clinical chemistry analysis. These rats were the ones housed in the metabolic cages and the urine collected during that time was subjected to urinalysis. Autopsies were conducted on all animals in the study and the following organ weights were recorded: adrenal glands, brain, heart, kidneys, liver, spleen, testes, thymus, and thyroid. Samples were collected and preserved for histological examination of the organs previously listed, an additional 35 sites, and any gross legions identified during autopsy.

No deaths or abnormal clinical signs were recorded during this study. A statistically significant decrease in body weight gain and food consumption was observed in the male and female rats administered the highest dose level (2.0%). In male animals the final mean body weight was approximately 5% lower than that of control animals and in female animals the body weight was approximately 9% lower. Transient decreases in water intake were also observed in the animals assigned to the highest dose group. Based on the reported daily feed intake, the associated intake of glutaminase concentrate was reported to be 0,

133, 388, and 1,239 mg/kg body weight/day in male rats and 0, 145, 450, and 1,432 mg/kg body weight/day in female rats.

No ophthalmologic, urinalysis and haematology changes were related to the glutaminase concentrate administration. Results of blood chemistry showed decreased plasma alkaline phosphatase activity (males, mid and high-dose group / females, high-dose group), alanine aminotransferase activity (males, mid and high-dose group / females, high-dose group) and level of chloride (females, mid-dose group). At the highest-dose group, decreases of the absolute weight of the brain (female), spleen (females), adrenals (females) and liver (males and females) and increases of the relative weights of the testes (males, of course) and brain (females) were observed. Gross pathology and histopathology showed no dose-related changes.

The changes in the growth of the animals in the highest dose group occurred in the absence of any signs of toxicity and was associated with decreased food consumption and therefore, potentially related to the palatability of the diet. However, it is not possible to determine that this is absolutely true and therefore the no-observed-adverse-effect level (NOAEL) for this study was determined to be the second highest dose level tested, 0.6% of the diet. On a body weight basis, this level of intake was determined to be equivalent to 388 mg/kg body weight/day in male rats and 450 mg/kg body weight/day in female rats.

C.2.1.3 Summary of Toxicity Data

The glutaminase concentrate employed in the production of Glutaminase SD-C100S preparations has been demonstrated to have no mutagenic or genotoxic properties. No signs of toxicity were reported in the 13-week repeated dose toxicity study conducted in male and female Wistar rats; however, due to a decrease in growth observed at the highest group the NOAEL for this study was determined to be the second highest dose tested. On a body weight basis, this level of intake was determined to be equivalent to 388 mg/kg body weight/day in male rats and 450 mg/kg body weight/day in female rats.

C.2.2 Sodium Chloride

As previously mentioned, the glutaminase concentrate is diluted with NaCl in order to produce the final enzyme preparation. The proposed maximum use level for the final enzyme preparation, 0.2%, could potentially result in a small increase in the salt content of the finished food products if the manufacturer does not or cannot adjust the overall salt content of the food. Based on the proposed levels of use, the inclusion of Glutaminase SD-C100S in a food will provide 0.182 g NaCl per 100 g of food while Glutaminase C100S would provide 0.19 g NaCl per 100 g of food. This information will be clearly displayed in the technical data sheets supplied to food manufacturers employing SD-C100S in their production process. Sodium chloride is considered by Food Standards Australia New Zealand (FSANZ) to be a food and is the subject of Standard 2.10.2 in the Australia New

Zealand Food Standards Code (FSANZ, 2014). The sodium chloride employed in the production of Glutaminase SD-C100S meets the specifications for salt listed in Standard 2.10.2. The Standard includes no limitations on the use of sodium chloride in food and therefore, no further data is present to support the safety of this ingredient.

C.3 Information on Potential Allergenicity

C.3.1 Source of the Processing Aid

B. amyloliquefaciens is used and approved for use as a source organism for processing aids in Australia and New Zealand (FSANZ, 2014). No allergenicity warnings are associated with the use of this organism in foods in Australia and New Zealand. A review of the published literature failed to identify reports of allergic reactions to *B. amyloliquefaciens* or to currently approved enzymes derived from *B. amyloliquefaciens*. Since 1992, no adverse effects have been reported in workers exposed to *B. amyloliquefaciens* GT2.

C.3.2 Residual Allergens from the Culture Medium

Although soybean oil and defatted soybean is used in the fermentation medium used in the preparation of the glutaminase enzyme (see Section A.4.1), residual soy allergens are not expected to be present in the final commercial product. The Glutaminase SD-C100S is proposed for use at 0.2% during the manufacture of certain seasonings, which is equivalent to 0.018% of the glutaminase concentrate. Given that the glutaminase enzyme concentrate is added only at low levels (0.018%) to certain food ingredients (*e.g.,* yeast extracts, hydrolysed vegetable proteins, hydrolysed animal protein, soy sauce, miso, vinegar, fish sauce, *etc*.), which are then subsequently added to food products at low levels for seasoning purposes, the exposure to any potential residual soy allergens in final food products consumed will be negligible and extremely unlikely to be of any allergenic concern.

C.3.3 Allergenicity of Glutaminase

A literature search on Toxnet and Pubmed databases was conducted to identify allergenic reactions to glutaminase enzymes. This intensive literature search failed to find any scientific work on this effect.

An assessment of the allergenic potential was conducted using the Allergen Database for Food Safety (ADFS), the results of which are presented in Appendix C. The results indicate that glutaminase from *B*. *amyloliquefaciens* is of low concern with regard to allergenicity with the amino acid sequence from the glutaminase matching none of the allergens contained in the database.

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

Bacillus amyloliquefaciens is used and approved for use as a source organism for food products and processing aids in numerous jurisdictions including Australia and New Zealand (FSANZ, 2014). In 1999, the FDA established that carbohydrase and protease enzyme preparations derived from either *B. amyloliquefaciens* or *B. subtilis* were considered Generally Recognized as Safe (GRAS) for use as a direct food additive (U.S. FDA, 1999).

B. amyloliquefaciens GT2 has been employed in the production of Glutaminase SD-C100S since 2008. Recently, SD-C100S received approval for use as a processing aid in the manufacture of hydrolysed proteins and yeast extracts in France (AFSSA, 2009). As a component of this approval, AFSSA evaluated the technical function, manufacturing process, and safety of SD-C100S. AFSSA concluded that the proposed uses for Glutaminase SD-C100S did not present a health risk to the consumer and rendered a favourable opinion on its use.

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

D.1 Information on the Source Microorganism

The parent micro-organism used for the glutaminase production is classified as *B. amyloliquefaciens* strain NP. The isolate employed by Amano Enzyme to produce glutaminase (Strain GT2) is a mutant which is derived from the NP strain by conventional process mutation using N-methyl-N'-nitro-N-nitrosoguanidine and the strain is not genetically modified. The taxonomic classification of *B. amyloliquefaciens* is presented below.

Super Kingdom	*Bacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Bacillaceae*
Genus	*Bacillus*
Species	*amyloliquefaciens*

It should be noted that the parent strain (NP) was previously classified as *Bacillus subtilis,* a species closely related to *B. amyloliquefaciens* and for which there is also an extensive history of use as a source organism for food products and processing aids in numerous jurisdictions, including Australia and New Zealand (FSANZ, 2014). The parent and derivative *Bacillus* strains used in the production of glutaminase (NP and GT2, respectively) have been characterized by TNO Nutrition and Food Research Institute, and using DNA:DNA hybridization, it has been confirmed that the GT2 strain is *B. amyloliquefaciens* according to the Bergey's Manual of Systematic Bacteriology (see Appendix D). The *B. amyloliquefaciens* GT2 strain is available for examination from Amano Enzyme Inc, Gifu R & D Center, Japan.

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

According to the risk classification derived by the European Community to protect workers exposed to biological agents, *B. amyloliquefaciens* would be classified in Group 1 as a biological agent that is unlikely to cause human disease (EC, 2000). An intensive bibliographic search, using several data bases including Chemical Abstract Series, Life Science Collection, Medline, Toxline and Biosis Previews, was performed in order to determine published toxic, mutagenic or pathogenic properties of *B. amyloliquefaciens*. Two publications were identified in which *B. amyloliquefaciens* was reported to be non-cytotoxic and non-pathogenic (Osipova *et al.*, 1998; Pedersen *et al.,* 2002).

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

As previously mentioned, the source micro-organism is neither genetically modified nor self cloned. The isolate used to produce glutaminase (Strain GT2) is a mutant which derived from the NP strain by conventional mutation using N-methyl-N'-nitro-N-nitrosoguanidine (Appendix D). 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 (GT2) is fermented for 24 hours and is divided into 0.5 mL vials (200 vials are prepared for a single batch). They are kept at -80°C in a locked freezer. When ready, a single vial is used for each 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

Glutaminase prepared from *B. amyloliquefaciens* is typically used as a processing aid for the hydrolysis of L-glutamine into L-glutamic acid in the production of certain food ingredients used as seasonings. As a result, the proposed food uses for glutaminase derived from *B. amyloliquefaciens* include the following food categories:

- Yeast extracts
- Hydrolysed vegetable protein (HVP)
- Hydrolysed animal protein (HAP)
- Sauces and condiments (*e.g.*, soy sauce, miso, vinegar, fish sauce, *etc*.)

Glutaminase SD-C100S is proposed for use in these foods at a level of 0.2%, resulting in a use level of 0.018% for the glutaminase concentrate. Based on the information presented above and the groupings employed in the Australian National Nutrition Survey (NNS) the proposed food uses and use levels for are listed in Table F.1-1 along with the associated use levels for glutaminase.

Table F.1-1 Proposed Food Uses and Use Levels for Glutaminase

Food Grouping	Proposed Food Use	Proposed Use Level for Glutaminase
Savoury sauces and condiments	Gravies and savoury sauces	0.018%
	Pickles, chutneys and relishes	0.018%
	Salad dressings	0.018%
Miscellaneous	Yeast, vegetable and meat extracts	0.018%
	Herbs, spices, seasonings and stock cubes	0.018%

F.2 Anticipated Residue Levels of Glutaminase

The estimated residue levels of residue for the processing aid were based on the Total Organic Solids (TOS) percentage of glutaminase concentrate contained within Glutaminase SD-C100S. The TOS content of the glutaminase concentrate was assessed within 8 production scale batches of the concentrate. In order to determine the TOS content of the concentrate the ash, moisture, and diluents content of each batch were determined. The results of the analysis are summarised in Table F.2-1 and the original reports are presented in Appendix F.

Table F.2-1 Determination of Total Organic Solid (TOS) Content of Glutaminase Concentrate								
Parameters	**Batch Number**							
	GT7062 5L12[1]	**GT7061 3L12**[1]	**GT70515 L12**[1]	**GT7060 6L12**[1]	**GT7091 6L12**[2]	**GT81218 5.00UP**[2]	**GT50710 5.00UP**[2]	**GT04060 45.00UP**[2]
Ash (g/100)	3.3	3	5.3	3.7	3	11	10.5	7.3
Moisture (g/100g)	11.2	8.7	7.8	8.2	9.2	5.6	5.7	5.5
Diluent (g/100g)	0	0	0	0	0	0	0	0
TOS (%)[3]	85.5	88.3	86.9	88.1	87.8	83.4	83.8	87.2

[1] Batch analysis conducted in July 1988 by TNO
[2] Batch Analysis conducted in May 2005 by Japan Food Research Laboratories
[3] TOS = 100 – (Ash + Moisture +Diluent)

Based on the results presented in Table F.2-1, the TOS percentages were calculated according to the following formula:

TOS = 100 – (A + M + D)
Where A = ash content, M = moisture, and D = content of diluents

As previously mentioned the final Glutaminase SD-C100S product comprises 91% NaCl and 9% glutaminase concentrate. As a result, the estimated residual TOS levels were based on the minimum and maximum use levels for the glutaminase concentrate alone as opposed to the finished enzyme preparations. Based on the mean calculated TOS content (86.4%) and the proposed use levels for glutaminase listed in Table F.1-1, the anticipated residues for all foods in which glutaminase is proposed for use are presented in Table F.2-2.

Table F.2-2 Proposed Food Uses and Anticipated Maximum Residue Levels for Glutaminase		
Food Grouping	**Proposed Food Use**	**TOS Residue Level (mg/kg)** [a]
Savoury sauces and condiments	Gravies and savoury sauces	155.5
	Pickles, chutneys and relishes	155.5
	Salad dressings	155.5
Miscellaneous	Yeast, vegetable and meat extracts	155.5
	Herbs, spices, seasonings and stock cubes	155.5

Abbreviation: TOS = Total Organic Solid.
[a] Based on a maximum proposed use level of 0.018% for glutaminase and a mean TOS content of 86.4% for the glutaminase concentrate

F.3 Information on the Likely Level of Consumption of Glutaminase SD-C100S and Glutaminase

The NNS was conducted to assess the dietary intakes of Australians in 1995. Individuals aged 2 years and older completed 24-hour diet recall questionnaires to assess food and beverage intake and well as additional questionnaires to determine the usual frequency of intake, food-related habits and attitudes, and physical measurements. Food categories that

correspond to the proposed food uses for glutaminase were identified within the survey. The mean intakes of these food categories as reported in the published results of the NNS are presented in Table F.3-1. The intake of each individual food category is summed in order to estimate the total potential intake of all foods in which glutaminase is proposed for use.

Table F.3-1 Consumption of Foods in which Glutaminase is Proposed for Use (NNS, 1995)[a]

Population group	Mean Intake of Individual Food Groups (g/day)					Total Mean Proposed Food Use Intake (g/day)
	Gravies and savoury sauces	Pickles, chutneys and relishes	Salad dressings	Yeast, vegetable, and meat extracts	Herbs, spices, seasonings and stock cubes	
Males						
2 to 3 years	9.3	0.3*	0.2*	0.8*	na	10.6
4 to 7 years	12.9	0.3*	1.4	1.4	na	16.0
8 to 11 years	18.8	1.2*	1.3	1.7	na	23.0
12 to 15 years	24.4	1.6*	2.7	1.9	na	30.6
16 to 18 years	36.1	1.2*	3.7	1.1	na	42.1
19 to 24 years	30.0	1.4	2.9	1.5	0.1*	35.9
25 to 44 years	30.9	3.1	3.0	1.2	0.2*	38.4
45 to 64 years	22.5	3.6	3.3	0.7	0.1*	30.2
65 and over	19.3	2.8	3.1	0.7	0.1*	26.0
Females						
2 to 3 years	8.4	0.4*	0.7*	1.5*	na	11.0
4 to 7 years	9.7	0.7*	1.0	1.2*	na	12.6
8 to 11 years	14.1	0.6*	1.3	1.4*	na	17.4
12 to 15 years	22.1	1.5*	2.3	1.9*	na	27.8
16 to 18 years	24.5	0.7*	2.5	0.7*	0.1**	28.5
19 to 24 years	24.1	1.6	3.3	0.7	0.1*	29.8
25 to 44 years	21.8	2.0	3.2	0.6	0.2*	27.8
45 to 64 years	19.3	2.0	3.7	0.6	0.1*	25.7
65 and over	14.8	2.0	2.8	0.7	0.1*	20.4

Abbreviation: na = not available

[a] Data was taken from the 1995 Australia Nutrition Survey. Estimates followed by an asterisk (*) have a relative standard error (RSE) between 25 and 50%, and thus is considered to be subject to high standard errors and should be used with caution. Estimates followed by double asterisks (**) have RSEs greater than 50%, and are considered too unreliable for general use (NNS, 1995).

In order to estimate the anticipated exposure to TOS resulting from the proposed uses of the glutaminase employed in the production of Glutaminase SD-C100S, the mean body weight for each population group were identified within the published results of the survey. The total intake of foods in which glutaminase is proposed for use was then multiplied by the maximum anticipated residual TOS levels (155.5 mg/kg, Table F.2-3) in order to estimate the likely maximum TOS intake. The results of the assessment of exposure based on the 1995 NNS survey are presented in Table F.3-2.

Table F.3-2 Anticipated Level of Exposure to TOS Resulting from the Proposed Food Uses of Glutaminase (NNS, 1995)				
Population group	**Reported Mean Body Weight (kg)**	**Total Mean Proposed Food Use Intake (g/day)**	**Maximum Anticipated Intake of TOS from Proposed Use of Glutaminase**	
			mg/day	**mg/kg bw/day**
Males				
2 to 3 years	15.5	10.6	1.65	0.11
4 to 7 years	22.3	16.0	2.49	0.11
8 to 11 years	34.6	23.0	3.58	0.10
12 to 15 years	56.5	30.6	4.76	0.08
16 to 18 years	72.3	42.1	6.55	0.09
19 to 24 years	78.3	35.9	5.58	0.07
25 to 44 years	82.4	38.4	5.97	0.07
45 to 64 years	84.4	30.2	4.70	0.06
65 and over	78.6	26.0	4.04	0.05
Females				
2 to 3 years	15.3	11.0	1.71	0.11
4 to 7 years	22.4	12.6	1.96	0.09
8 to 11 years	36.7	17.4	2.71	0.07
12 to 15 years	54.5	27.8	4.32	0.08
16 to 18 years	61.4	28.5	4.43	0.07
19 to 24 years	63.4	29.8	4.63	0.07
25 to 44 years	67.3	27.8	4.32	0.06
45 to 64 years	71.2	25.7	4.00	0.06
65 and over	66.1	20.4	3.17	0.05

Abbreviations: bw = body weight; TOS = Total Organic Solid.

Based on the total intake of foods in which glutaminase is proposed for use, the absolute estimated mean daily exposure to TOS within the general Australian population was estimated to fall between 1.65 and 6.55 mg/day. On an absolute basis the intake of TOS were estimated to be greatest in male teenagers between the ages of 16 and 18 years and lowest in male children 2 to 3 years of age. On a body weight basis, the daily intake of TOS within the general population was estimated to range between 0.05 and 0.11 mg/kg body weight/day. The highest estimated levels of intake of TOS were observed to occur in children between 2 and 7 years of age while the lowest intakes were observed to occur in elderly male and female adults (65 years of age and older).

A similar assessment was also conducted with the data from the NNS conducted in 2007 for children between the ages of 2 and 16 years of age (NCNPAS, 2007). Similar to the 1995 data, a combination of 24-hour diet recalls and food frequency questionnaires (FFQ) were employed in order to estimate the intake of foods across Australia. The published results available for this survey include fewer details than those published for the 1995 survey, and therefore, only the major food categories were available for use in the estimate of TOS exposure resulting from the proposed food uses of glutaminase. The intake of the relevant

foods categories, including all foods within the miscellaneous and savoury sauces and condiments designations, are presented in Table F.3-3.

Table F.3-3 Consumption of Foods by Children in which Glutaminase SD-C100S is Proposed for Use (NCNPAS, 2007)

Population Group	Mean Intake of Individual Food Groups (g/day)		Total Mean Proposed Food Use Intake (g/day)
	Savoury Sauces and Condiments	Miscellaneous	
Males			
2 to 3 years	12.0	5.1	17.1
4 to 8 years	15.8	2.8	18.6
9 to 13 years	31.8	5.9	37.7
14 to 16 years	35.1	3.6	38.7
Females			
2 to 3 years	10.5	5.2	15.7
4 to 8 years	15.0	4.7	19.7
9 to 13 years	26.0	3.3	29.3
14 to 16 years	30.6	4.7	35.3

In order to estimate the anticipated exposure to TOS resulting from the proposed uses of glutaminase, the mean body weight for each population group were identified within the published results of the 2007 survey (NCNPAS, 2007). The intake of all foods within the overall categories in which glutaminase is proposed for use was then multiplied by the maximum anticipated TOS levels (155.5 mg/kg, Table F.2-3) in order to estimate the likely intake. The results of the assessment of exposure based on the 2007 NNS survey are presented in Table F.3-4.

Table F.3-4 Anticipated Level of Exposure to TOS Resulting from the Proposed Food Uses of Glutaminase (NCNPAS, 2007)

Population group	Reported Mean Body Weight (kg)	Total Mean Proposed Food Use Intake (g/day)	Maximum Anticipated Intake of TOS from Proposed Inclusion of Glutaminase SD-C100S	
			mg/day	mg/kg bw/day
Males				
2 to 3 years	15.8	17.1	2.66	0.17
4 to 8 years	24.3	18.6	2.89	0.12
9 to 13 years	44.9	37.7	5.86	0.13
14 to 16 years	65.1	38.7	6.02	0.09
Females				
2 to 3 years	15.2	15.7	2.44	0.16
4 to 8 years	23.9	19.7	3.06	0.13
9 to 13 years	45.4	29.3	4.56	0.10
14 to 16 years	60.2	35.3	5.49	0.09

Abbreviations: bw = body weight; TOS = Total Organic Solid.

Based on the total intake of the food categories in which glutaminase is proposed for use the absolute estimated mean daily exposure to TOS in children aged 2 to 16 years was estimated to range between 2.44 and 6.02 mg/day. Within the age groups surveyed the largest intakes of TOS were estimate to occur in male teenagers between 14 and 16 years of age and the lowest were estimated to occur in female children between 2 and 3 years of age. On a body weight basis, the highest estimated level of intake of TOS was observed to occur in male children 2 and 3 years of age at 0.17 mg/kg body weight/day and the lowest levels of intake were observed to occur in male and female teenagers 14 to 16 years of age at 0.09 mg/kg body weight/day. These estimates represent a worst-case scenario for the intake of TOS from the proposed food uses of glutaminase. The intakes were calculated assuming that all foods within the gravies and sauces and miscellaneous contraries are produced using the highest recommended level of glutaminase concentrate. Furthermore, it is assumed that all foods are produced using glutaminase and that all glutaminase employed comes from Glutaminase SD-C100S which has the higher glutaminase concentrate content.

Two (2) dietary intake surveys were identified in which the consumption of foods were examined within residents of New Zealand. Within the 1997 NNS the proposed food uses for glutaminase are primarily categorised as sauces and therefore, this food category was employed to represent the possible food intakes of Glutaminase SD-C100S (New Zealand NNS, 1997). Within the 2002 children's survey conducted in New Zealand, the proposed food uses for Glutaminase SD-C100S were primarily categorised as savoury sauces and condiments (New Zealand NCNS, 2002). Neither survey provided data on the amount of food consumed in these categories, rather the frequency of consumption or the contribution to energy and nutrient intake was presented.

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

Glutaminase enzymes are currently not permitted for use as a processing aid by FSANZ (2013), and therefore, it is conceivable that those manufacturers who produce yeast extracts, hydrolysed vegetable and animal protein may choose to use the Glutaminase SD-C100S from Amano Enzyme. In Japan, a total of 9 soy sauce breweries and 38 miso production facilities are known to employ glutaminase preparations in their production processes (Amano Enzyme, 2005).

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

Glutaminase SD-C100S is approved for us in both Japan and France. In both jurisdictions 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

The intake of foods in which Glutaminase SD-C100S is proposed for use are not anticipated to have changed drastically in the time since the most recent intake survey was conducted. This assumption is based on the results of the 1995 intake survey compared to those from the 2007 survey. Within similar age groups the intakes of foods in which Glutaminase SD-C100S is proposed for use were found to be similar between the 2 surveys despite the fact that not all food groups included in the savoury sauces and miscellaneous categories were applied to the estimates of consumption derived from the 1995 survey data.

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