Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include Oryzin (Protease) from *Aspergillus melleus* as a Processing Aid

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GENERAL REQUIREMENTS

1.0 APPLICANT DETAILS

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

2.0 PURPOSE OF THE APPLICATION

The purpose of the application is to amend Schedule 18 of the Food Standards Code to permit the use of Oryzin (Protease) from *Aspergillus melleus* as a processing aid intended for use in baking, dairy processing, egg processing, meat and fish processing, protein processing, yeast processing and flavoring production.

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 Oryzin (Protease) derived from *Aspergillus melleus* in the Australia New Zealand Food Standards Code as a processing aid will allow producers to add Oryzin (Protease) step to their production process. There will be no additional cost to the regulator if the processing aid is approved as the use of Oryzin (Protease) derived from *Aspergillus melleus* will not impact the regulation of these food products.
Oryzin (Protease) acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

The effect of the enzymatic conversion with the help of Oryzin (Protease) is the conversion of the substrate proteins and peptides in various proteinic food raw materials, which may result in improvement of organoleptic properties (taste and flavor), physiological properties (foamability, emulsifying ability, heat stability, viscosity) and nutritional properties (absorptivity, digestivity).

3.1.1.2 Impact on International Trade

The approval of Oryzin (Protease) derived from *Aspergillus melleus* as a processing aid may, in the future, promote international trade and reduction of technical barriers to trade, while continuing to protect public health and safety.

4.0 INFORMATION TO SUPPORT THE APPLICATION

Sections A through F of this application contain detailed data that supports the quality, efficacy, and safety of Oryzin (Protease) derived from *Aspergillus melleus* 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 Oryzin (Protease) derived from *Aspergillus melleus* 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 Oryzin (Protease) derived from *Aspergillus melleus*, 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 Oryzin (Protease) 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 Oryzin (Protease) 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 Oryzin (Protease) derived from *Aspergillus melleus* has a history of use in Denmark, France, Canada, Japan and China. It is expected that the introduction of Oryzin (Protease) derived from *Aspergillus melleus* to the Australia/New Zealand market will be well received.

5.0 ASSESSMENT PROCEDURE

Amano Enzyme considers the most appropriate assessment procedure for the application herein, which relates to an amendment Schedule 18 of the Food Standards Code to include Oryzin (Protease) derived from *Aspergillus melleus* as a processing aid, to be the General Procedure (Subdivision D), Cost Category Level 1 (up to 350 hours). This is based on the fact that FSANZ approved processing aids derived from assessed and granted approval to similar enzymes, Aspergillopepsin I (EC3.4.23.6) and Aspergillopepsin II (EC3.4.23.19). Also, Amano Enzyme's oryzin product has already been approved and marketed in several other major jurisdictions for food uses that are similar to those proposed in Australia/New Zealand.

6.0 CONFIDENTIAL COMMERCIAL INFORMATION

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

7.0 EXCLUSIVE CAPTURABLE COMMERCIAL BENEFIT (ECCB)

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

> An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food

regulatory measure under Section 22 if:

a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that would be prepared in relation to the application; and

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

8.0 INTERNATIONAL AND NATIONAL STANDARDS

The following national and international standards are relevant to the current application:

- Protease is listed on the Food Additive Index of CODEX General Standard for Food Additives (GSFA) (INS: 1101(i)). Also, this food enzyme, Oryzin (Protease), complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006). (See also A.5.1)
- Protease (exopeptidase) from *Aspergillus melleus* is approved in France and Denmark.
- Protease from *Aspergillus melleus* is on the “List of Existing Food Additives” published by the Ministry of Health and Welfare of Japan (MHLW, 2014).
- Protease from *Aspergillus melleus* is approved as a food additive in China.
- Protease from *Aspergillus melleus* is on a list of Permitted Food Enzymes of Health Canada.

9.0 STATUTORY DECLARATION

A signed statutory declaration is appended to this application.

10.0 CHECKLIST

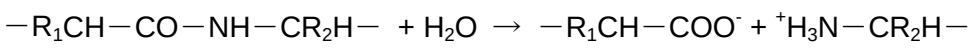
A completed checklist relating to the information required for submission is appended to this application.

SECTION A: TECHNICAL DESCRIPTION OF Oryzin (Protease)

Oryzin (Protease) 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

Oryzin (Protease) is powdered enzyme and is an enzyme catalyzing the hydrolysis of proteins broad specificity. The general reaction scheme is:



Amano Enzyme has prepared Oryzin (Protease) enzyme preparation that is derived from *Aspergillus melleus* by means of a fermentation process. The enzyme intended for use as a processing aid in food. A full description of the manufacturing procedures is provided in Section A.4.

Based on the foregoing description, protease derived from *Aspergillus melleus* would fall under the following classification within Schedule 18 (Processing Aids):

18-4 (5) Permitted enzymes of microbial origin

The maximum proposed level of Oryzin (Protease) to food products use is 0.14%.

A.2 Information on the Identity of the Processing Aid

Common name:	Protease
Systematic name:	Oryzin
EC number:	EC 3.4.21.63
CAS registration number:	9074-07-1
EINECS number:	232-977-6

The Amano's Oryzin (Protease) is produced by *Aspergillus melleus* strain P-52. Strain P-52 is not genetically modified organism but a chemically mutated production strain derived from the original strain No.26st (See also section D.1). *Aspergillus melleus* has been used for many years for food or feedstuffs purposes or in the production of enzymes processing aids in Denmark, France, Canada, Japan and China.

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 Oryzin (Protease) Activity

An analytical method for the detection and quantification of Oryzin (Protease) activity is presented in Appendix A - 1. In brief, protease activity can be obtained by the colorimetric measurement, making use of Folin's reaction, of the amount of acid-soluble low-molecular products, which is increased owing to the hydrolysis of the peptide linkages when protease acts on casein. One protease activity unit is the amount of enzymes that produces Folin's TS-colorable substrate equivalent to 1µg of tyrosine per minute under the conditions described in the Appendix.

A.3.1.2 Characterization of Oryzin (Protease) Activity

The technical function of Oryzin (Protease) is to catalyze the hydrolysis of proteins broad specificity.

The effects of temperature and pH on the activity of the Oryzin (Protease) concentrate were examined and the results are presented in Figures A-1 and A-2. 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 Oryzin (Protease) solution. The effect of temperature and pH on the activity were compared to the activity measured under standard conditions. For the assessment of the impact of temperature on activity, the standard conditions were considered to be a water bath temperature of 37°C. The activity of the sample at a given pH was compared to the activity measured when the reaction was run at a pH of 8.0. Based on the assays conducted, the peak activity of the Oryzin (Protease) occurs at 40-45°C and a pH of approximately 7-8.

Figure A - 1: Effect of the temperature on the activities

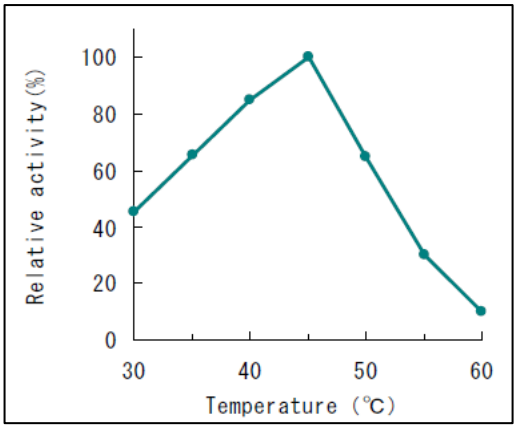
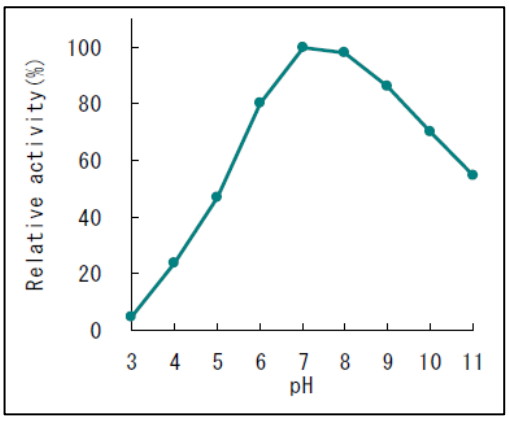


Figure A - 2: Effect of the pH on the activities



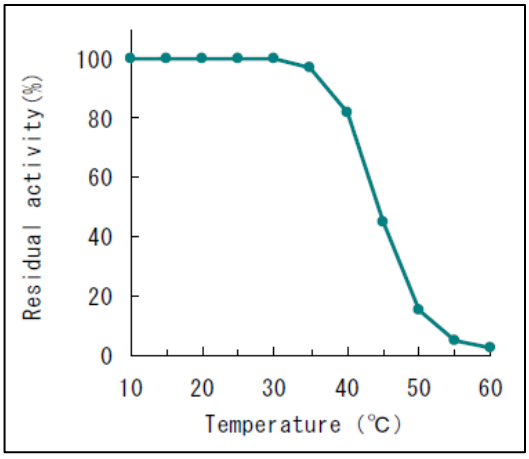
A.3.2 Stability

pH and THERMAL STABILITY

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

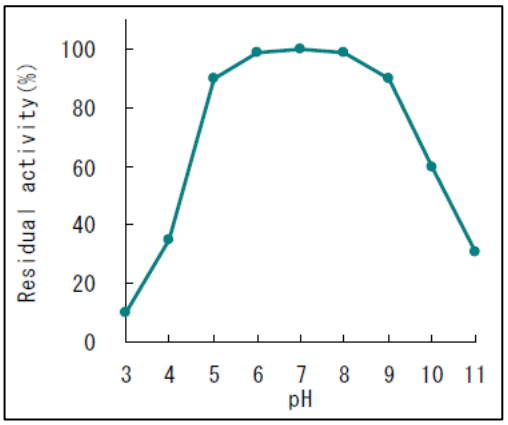
The results of the assessment of stability under varying temperature and pH conditions indicate that Oryzin (Protease) is stable at up to 50°C and in a pH range of 4 to 11.

Figure A - 3: Thermal stability of the activities



1% Enzyme solution, pH 8.0, 60 min incubation

Figure A - 4: pH stability of the activities



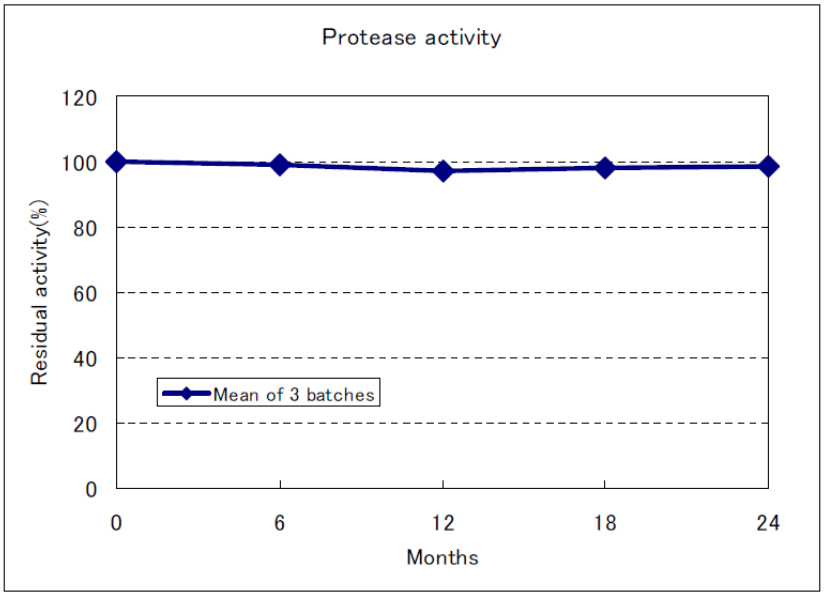
1% Enzyme solution, 37°C, 60 min incubation

LONG TERM STABILITY

The stability of the enzyme preparation was assayed by Amano Enzyme Inc. Samples were kept in the airtight bag at the room temperature. The protease activity was periodically measured for 24 months. Results are shown at the Figure A-5.

It could be concluded that the initial protease activity remained at least until 24 months at the room temperature.

Figure A - 5: The stability of the enzyme preparation



A.3.3 Possible Interactions with Food Constituents

Oryzin (Protease) is an enzyme which acts on single substrate and would therefore, not be expected to act on other constituents in the food. The enzyme preparation must be inactivated either by temperature or pH changes. Amano Enzyme recommends that the inactivation be accomplished by changing the pH of the food so that it is lower than 4 or greater than 11 or by increasing the temperature above 55°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 protein present in non-target foods.

A.3.4 Characterisation of Secondary Activities

As far as Amano Enzyme is aware, the Oryzin (Protease) described in this dossier does not possess any enzymatic side activities which might cause adverse effects.

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

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

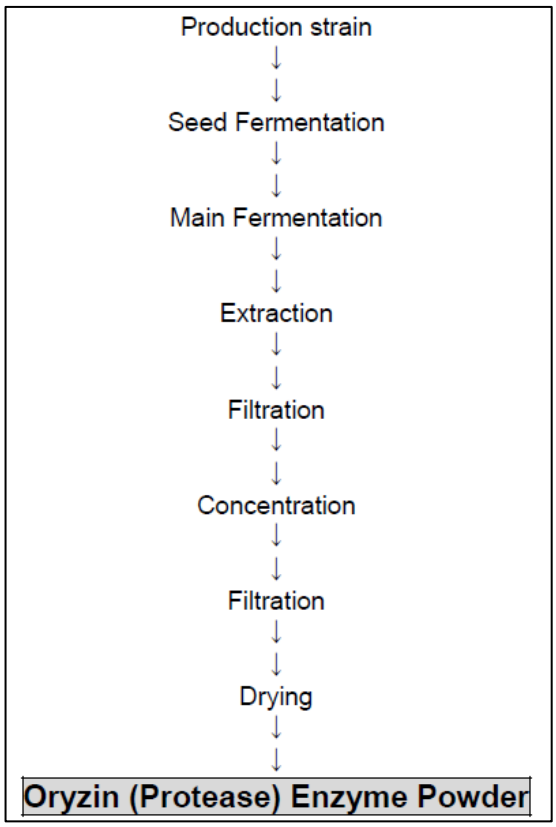
Furthermore, a wide range of food enzymes, including proteases and phospholipases, have been on the market for decades and have been approved on the market for use in food on basis of safety documentation.
Finally proteases and phospholipases are natural constituents of foods. For instance, bromelain is a protease that is ingested in its active form by consumers eating raw pineapples. Phospholipase is a normal constituent of wheat flour (Nolte *et al.,* 1974) and is one of the digestive enzymes present in the pancreatic juice of mammals, including humans (de Haas *et al*., 1968, Rossiter, 1968, Johnson and McDermott, 1974).

A.4 Manufacturing Process

A.4.1 Manufacturing Steps

A schematic overview of the overall manufacturing process for Oryzin (Protease) is provided in the Figure A-6.

Figure A - 6: Manufacturing Process



In brief, the production begins with the fermentation of *Aspergillus melleus* under standard culturing conditions. Recombinant DNA technology is not used to obtain this strain. Once the fermentation is complete, the enzyme is extracted with water from the fermentation media. The extracted solution containing the enzyme is then submitted to a series of separation and concentration steps at the end of which the food enzyme concentrate can be formulated into a commercial preparation that will be used in food processing.

The enzyme is produced according to the FSSC22000 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) for food additives certification and certificate of conformity to FSSC22000 are provided in Appendix A - 2.

A.4.2 Raw Materials

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

Table A - 1: Raw Materials and Processing aids Used in the Production

Substance	**Grade**	**Function**	**Status in Australia and New Zealand (FSANZ, 2014)**
Soybean flour	Food	Fermentation media	Food ingredients
Wheat bran	Food	Fermentation media	Food ingredients
Wheat flour	Food	Fermentation media	Food ingredients
Acetic acid	Food additives	pH adjustment	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Hydrochloric acid	Food additives	pH adjustment	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Sodium hydroxide	Food additives	pH adjustment	Permitted for use in the production of processing aids (Schedule 18)
Activated carbon	Food additives	Processing aid	Permitted for use in the production of processing aids (Schedule 18)
Calcium hydroxide	Food additives	Processing aid	Approved for use a food additive when used in accordance with GMP (Schedule 8)
Diatomaceous earth	Food additives	Filter aid	Permitted for use in the production of processing aids (Schedule 18)

A.4.3 Residual Allergens from the Culture Medium

Although soybean and wheat products are used in the fermentation medium, residual soy and wheat allergens are not present in Oryzin (Protease) enzyme powder (less than 3.0µg/g).

Furthermore, as described in Section F.1, Oryzin (Protease) is added only at low levels (0.14% at the maximal*) to food products for enzyme reaction. The exposure to any potential residual soy and wheat allergens in final food products consumed will be negligible and extremely unlikely to be of any allergenic concern.

*: Maximal use level to food product: 1,186 mgTOS/kg (See section F.1)
= 1,400mg/kg (TOS: 84.7%, see section A.5.2)
= 0.14 %

A.5 Specification for Identity and Purity

A.5.1 Product Specification

The specifications for Oryzin (Protease) are given in the Table A-2. It is proposed that the food enzyme Oryzin (Protease) should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006):

Table A - 2: Specification for Oryzin (Protease) enzyme powder

The Chemical and Microbiological Specification	
Lead	Not more than 5 mg/kg
Salmonella sp.	Absent in 25 g of sample
Total coliforms	Not more than 30 per gram
Escherichia coli	Absent in 25 g of sample
Antimicrobial activity	Not detected
Mycotoxins	No significant levels[1]
Enzyme Activity	
Protease activity	Not less than 380,000 u/g
General Properties	
Appearance	Off white to brown powder

A.5.2 Batch Analysis

The proof that the food enzyme Oryzin (Protease) complies with these specifications is shown by the analyses on various different batches. The results are provided in the Table A-3 and in Appendix A-3.

Protein content and relative purity of the food enzyme Oryzin (Protease) from *Aspergillus melleus* was measured, and the TOS values were calculated, in 3 batches. The result is shown in the Table A-3.

[1] See JECFA specifications, ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf, page 64: Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Table A - 3: Batch Analysis

Batch no	PZH-N67-006	PZH-N67-009	PZH-N67-011	Mean
Heavy metals				
Lead	0.03 mg/kg	0.03 mg/kg	0.03 mg/kg	-
Microbiology				
Salmonella sp.	ND/25g	ND/25g	ND/25g	-
Total coliforms	< 10cfu/g	< 10cfu/g	< 10cfu/g	-
Escherichia coli	ND/10g	ND/10g	ND/10g	-
Antimicrobial activity				
Antimicrobial activity	Negative	Negative	Negative	-
Mycotoxins				
Aflatoxin B1	ND	ND	ND	-
Aflatoxin B2	ND	ND	ND	-
Aflatoxin G1	ND	ND	ND	-
Aflatoxin G2	ND	ND	ND	-
Total Aflatoxin	ND	ND	ND	-
Ochratoxin A	ND	ND	ND	-
HT-2 Toxin (HT-2)	ND	ND	ND	-
T-2 Toxin (T2)	ND	ND	ND	-
Zearalenone (ZON)	ND	ND	ND	-
Sterigmatocystin	ND	ND	ND	-
Fumonisin FB1	ND	ND	ND	-
Fumonisin FB2	ND	ND	ND	-
Protein content and relative purity				
Ash (%)	9.4	9.3	9.2	9.3
Water (%)	5.6	6.0	6.4	6.0
TOS (%)	85.0	84.7	84.4	84.7
Enzyme activity (u/g)	849,000	808,000	846,000	834,000
Units/mg TOS	999	954	1003	985
Protein (%)	57.5	57.3	57.8	57.5

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 Oryzin (Protease) derived from *Aspergillus melleus.*

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

Protease P "Amano" 6SD-K, which is the commercial enzyme preparation and is composed of this Oryzin (Protease) enzyme powder and dextrin, has been accepted for use in protein hydrolysation of meat and vegetables, in cheese production as well as for production of meat- and yeast extracts in Denmark (Appendix C - 1).

Also, Protease (Exopeptidase) d'*Aspergillus melleus* is approved in France[2].

Protease 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).

Protease from *Aspergillus melleus* is permitted as a food additive that may be used as food enzymes in Canada and China (Appendix C - 2 and Appendix C - 3).

[2] http://www.legifrance.gouv.fr/affichTexteArticle.do;jsessionid=DAD5D03919D01BD2C81DBAD29173B47A.tpdila08v_1?idArticle=LEGIARTI000030251767&cidTexte=JORFTEXT000000271061&categorieLien=id&dateTexte=20150520

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

As mentioned in Section C.1, Oryzin (Protease) enzyme preparations have a wide history of use in food processing. To further support the safety of Oryzin (Protease) enzyme preparation, several toxicity studies have been conducted to assess the safety. The potential mutagenic and genotoxic activity of the Oryzin (Protease) 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 summary of the studies are as follows;

- Bacterial reverse mutation: No mutagenic activity under the given test conditions
- Chromosomal aberrations: No clastogenic activity under the given test conditions
- Systemic toxicity: The No Observed Adverse Effect Level (NOAEL) is 1,356 mgTOS/kg/day, which is the high dose in the study.

ABSENCE OF TOXINS

We tested for the presence of mycotoxins such as Aflatoxin B1, Ochratoxin A, Sterigmatocystin, Zearalenone and T-2 Toxin and confirmed their absence from the Oryzin (Protease). The results of search for mycotoxins are provided in the Table C-1 and in Appendix A-3.

Table C - 1: The results of Mycotoxins

Batch no	PZH-N67-006	PZH-N67-009	PZH-N67-011
Aflatoxin B1	ND	ND	ND
Aflatoxin B2	ND	ND	ND
Aflatoxin G1	ND	ND	ND
Aflatoxin G2	ND	ND	ND
Total Aflatoxin	ND	ND	ND
Ochratoxin A	ND	ND	ND
HT-2 Toxin (HT-2)	ND	ND	ND
T-2 Toxin (T2)	ND	ND	ND
Zearalenone	ND	ND	ND
Sterigmatocystin	ND	ND	ND
Fumonisin FB1	ND	ND	ND
Fumonisin FB2	ND	ND	ND

ND: Not detected

C.2.1 Oryzin (Protease) Concentrate

C.2.1.1 Mutagenicity and Genotoxicity

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

Bacterial Reverse Mutation Test in bacteria

Reference:

Study No: 45-998-05 (Appendix C - 4)

A bacterial reverse mutation test was conducted at Amano Pharmaceutical Co., Ltd. (Former Amano Enzyme Inc.)

The study was conducted in accordance with OECD guidelines (OECD, 1987) and guidelines of the Japanese Ministry of Health and Welfare (JMHW, 1985).

Summary:

Test article described in the report is Protease P “Amano”6. This product comprises Oryzin(Protease) concentrate diluted with potato dextrin to standardize enzyme activities. The test article (Lot No.PZQ05520) has a specific protease activity of 759,000u/g.

A reverse mutation test was conducted in 5 tester strains: *Salmonella typhimurium* (TA100, TA98, TA1535 and TA1537), and *Escherichia coli* strain WP2 uvrA. In this study, a dose-range finding test and main test were conducted with and without metabolic activation by the pre-incubation method. Four dose levels (5000, 1250, 313, 78 µg/plate) were set for the dose-range finding test and Five dose levels (5000, 2500, 1250, 625 and 313 µg/plate) for the main test. No biologically or statistically significant increases in the number of revertant colonies were observed in any tester strain, either in the absence or presence of metabolic activation.

It was therefore concluded that the test article does not induce gene mutations in the bacterial reverse mutation assay when tested under the test conditions employed for this study.

Chromosomal Aberration Test

Reference:

Study No: SBL 34-00 (Appendix C - 5)

Chromosome aberration in cultured Chinese hamster cells was conducted at SHIN NIHON BIOMEDICAL LABORATORIES, LTD.

The study was conducted in accordance with OECD guidelines (OECD, 1987) and guidelines of the Japanese Ministry of Health and Welfare (JMHW, 1988).

Summary:

The test article was protease enzyme preparation (Lot No.PZR08524) which has a specific protease activity of 999,000u/g.

CHL/IU cells, which were derived from the lungs of newborn female Chinese hamsters, were exposed *in vitro*, using the direct method test (22-hour and 46 hour treatments) and the metabolic activation method test (with and without S9 Mix). The dose levels used in the chromosomal aberration test were 19.5, 39.1, 78.1 and 156.3 µg/mL (Direct method test: 22-hour treatment); 4.9, 9.8, 19.5 and 39.1 µg/mL (Direct method test: 46-hour treatment); 78.1, 156.3, 312.5 and 625 µg/mL (metabolic activation method test: with S9 mix (recover time 16 hours) and without S9mix (recovery time 16 hours) ; and 78.1, 156.3, 312.5 and 625 µg/mL.

Also no statistically significant increase was noted in the frequency of structural aberrations or numerical aberrations in any treatment test irrespective of the presence or absence of a metabolic action system with S9-mix. From above-mentioned result, the test article did not show chromosomal aberration.

C.2.1.2 Repeat Dose Toxicity Assay

Acute, subacute and chronic toxicity tests (Appendix C - 6)

The test article was a protease enzyme preparation (Lot No. PZ4810N) which has a specific protease activity of 806,000 u/g. Test article described in the report is Protease P “Amano” 6. This product comprises Oryzin (Protease) concentrate diluted with potato dextrin to standardize enzyme activities.

Acute Toxicity Test

Male and female mice (ddy/S) and rats (Wister) were assigned to each group consisting of 10 animnals. Three administration routes were employed namely, oral (gavage), subcutaneous (injection) and intraperitoneal (injection). The LD50 values and their 95% confidence limits were calculated by Van der Waerden method.

The results were indicated below.

Mice

Route	LD50 mg/kg (95% confidece limit)	
	Male	Female
Oral	15,900 (15,000- 16,800)	17,300 (16,500-18,200)
Subcutaneous	109.5 (105.4-113.8)	109.5 (105.1-114.2)
Intraperitoneal	64.9 (61.8-68.2)	64.1 (61.0-67.4)

Rats

Route	LD50 mg/kg (95% confidece limit)	
	Male	Female
Oral	17,800 (17,600-18,000)	14,400 (14,100-14,700)
Subcutaneous	82.9 (73.6-93.5)	103.2 (92.0-115.7)
Intraperitoneal	62.9 (55.8-71.0)	65.3 (57.9-73.6)

Subacute Toxicity Test

Male and female rats (Wister) were assigned to each group consisting of 10 animals. The test article was administred orally by gavage for 30 days. Based on the results of acute toxicity test (gavage), three dose levels, 200, 1000 and 5000 mg/kg and the control (0 mg/kg) were employed. General conditions were observed daily and body weight, food and water consumption were measured every 5 days. Uninalysis were conducted after the final administration. After the administration period was finished, hematological and serum analysis, macroscopic analysis of organs, absolute and relative organ weight mesure and histopathological examination were executed.

A slight inhibition of body weight gains was observed in the group receiving 5000 mg/kg of male rats. No difference was found between the control and the treated groups of both male and female rats regarding general symptoms, food and water consumptions, hematological analysis, serum analysis, urinalysis, absolute and relative organ weight, macroscopic and histopathological examination.

Chronic Toxicity Test

Male rats (Wister) were assigned to each group consisting of 10 animals. The test article was administered orally with powder food for 26 weeks. Based on the results of subacute toxicity test, three dose levels, 500, 1000 and 2000 mg/kg and the control (0 mg/kg), were employed. Throughout the administration period, general conditions and food consumptions were measured at every 7 day. Hematological analysis and urinalysis were carried out at the 12th and 26th weeks of the test period. Serum analysis, macroscopic analysis and histopathological analysis were performed at the end of test period in the same manner as the subchronic toxicity test.

No difference was found between the control and the treated groups with regard to general conditions, body weight, food and water consumption, serum analysis, absolute and relative organ weight, macroscopic and histopathological examination.

In the hematological analysis and urinalysis, no difference was found between the control and treated groups on all points of tested (12th and 26th weeks).

As indicated above, maximum safety dose was considered to be 2000 mg/kg or more.

TOS OF THE MATERIAL USED FOR THE SYSTEMIC TOXICITY STUDY

Batch No.	PZ 4810N
Ash (%)	9.02
Water (%)	5.14
Dilution (%)	18.0
TOS (%)	**67.8**

SAFETY MARGIN

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). As was shown in Section F.3, the Total TMDI of the food enzyme is 1.725 mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 1,356 / 1.725 = 786

As is explained in Section F.3, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

C.3 Information on Potential Allergenicity

C.3.1 Source of the Processing Aid

Aspergillus melleus is used and approved for use as a source organism in many countries (refer to the section 8.0). No allergenicity warnings are associated with the use of this organism in food in these countries. Amano's *Aspergillus melleus* in this submission has been used safely for the production of food enzymes for over 35 years. Meanwhile no pathogenic or toxic accident has been arisen in the workers exposed to the strain.

C.3.2 Allergenicity of Oryzin (Protease)

Residual Allergens from the Raw Materials

Please refer to the section A.4.3.

Amino-acid sequence

The amino-acid sequence for the Oryzin (Protease) enzyme protein has been determined as indicated below.

1	ALTTQTGATW	GLGSISHKGE	SSTSYVYDSS	AGEGTYGYVV	DTGINVDHSE	50
51	FGGRASLAYN	AVGGQHVDSV	GHGTHVAGTI	GGKTYGVSKK	ANLLSVKVFQ	100
101	GESSSTSIIL	DGYNWAANDI	VSKSRTGKAA	INLSLGGGYS	YAFNQAVENA	150
151	FDEGVLTVVA	AGNENSDAGN	TSPASAPNAL	TVAASTNRNA	RASFSNYGSV	200
201	VDVFAPGQDI	KSAWIGGSSA	TNTISGTSMA	TPHIVGLAIY	LQALEGLTSP	250
251	AAVTKRIKEL	ATSGVVTDVK	GSPNLLAYNG	AA		282

The mature amino acid sequence of this enzyme protein is consisted of 282 and the Molecular Mass of this enzyme protein by the calculation from the sequence is about 28.5 kDa.

The homology search of the EFSA CEF Guidance document on food enzymes (EFSA, 2009b) could be performed. The results indicated that several proteases and protease related fragments are hit with Oryzin (Appendix C - 7). However these known allergens cause allergy via inhalation (Matsumura, 2012[3]).

[3] http://www.hindawi.com/journals/ja/2012/903659/

Literature Search

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

- The allergenic potential of enzymes was studied by Bindslev-Jensen et al. (2006) and reported in the publication: "Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry". The investigation comprised enzymes produced by wild-type and genetically modified strains as well as wild-type enzymes and Protein Engineered variants and comprised 400 patients with a diagnosed allergy to inhalation allergens, food allergens, bee or wasp. It was concluded from this study that ingestion of food enzymes in general is not likely to be a concern with regard to food allergy.
- Previously, the AMFEP Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products (Dauvrin et al., 1998). The overall conclusion was that – as opposed to exposure by inhalation – there are no scientific indications that the small amounts of enzymes in food can sensitize or induce allergy reactions in consumers.
- Enzymes when used as digestive aids are ingested daily, over many years, at much higher amounts when compared to enzymes present in food (up to 1 million times more). Wüthrich (1996) published a list of enzymes used as digestive aids and concluded that they are not potent allergens by ingestion.

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

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

- The food enzyme is used in small amounts during food processing, resulting in very small amounts of the enzyme protein in the final food. A high concentration generally equals a higher risk of sensitization, whereas a low level in the final food equals a lower risk (Goodman et al., 2008).
- In the case where proteins are denatured, the tertiary conformation of the enzyme molecule is destroyed. In general, these alterations in conformation are associated with decrease in the antigenic reactivity in humans: in the vast majority of investigated cases, denatured proteins are much less immunogenic than the corresponding native proteins (Valenta and Kraft, 2002; Valenta, 2002; Takai et al., 1997; Takai et al., 2000; Nakazawa et al., 2005; Kikuchi et al., 2006).
- In addition, residual enzyme proteins still present in the final food will be subjected to

digestion in the gastro-intestinal system, which reduces further the risk of enzyme allergenicity. While stability to digestion is considered as a potential risk factor of allergenicity, it is believed that small protein fragments resulting from digestion are less likely to be allergenic (FAO/WHO, 2001; Goodman et al., 2008).

- Finally, enzymes have a long history of safe use in food processing, with no indication of adverse effects or reactions. Moreover, a wide variety of enzyme classes (and structures) are naturally present in food. This is in contrast with most known food allergens, which are naturally present in a narrow range of foods.

Long History of Use

Furthermore, the enzyme has a long history of safe use in food processing. No adverse effects have been reported in worker exposed to the source strain or enzyme preparation, or consumer. The sales volume for food use for the last decade is indicated in the Table C-2.

Table C - 2**: Sales history of Protease from *Aspergillus melleus* for food use for the last decade**

Year	Volume (kg)	Cumulative Total (kg)
2002	9,012	9,012
2003	8,547	17,559
2004	9,715	27,274
2005	6,058	33,331
2006	18,966	52,297
2007	14,194	66,491
2008	11,771	78,262
2009	13,570	91,832
2010	13,607	105,439
2011	14,915	120,354

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

Protease from *Aspergillus melleus* (Commercial name: Protease P “Amano” 6SD) was evaluated in Denmark. In 2013, the enzyme received the approval for use. (See also Section C.1)

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

D.1 Information on the Source Microorganism

The production organism for this enzyme preparation is a strain of *Aspergillus melleus*
The wild type strain, *Asperergillus*, so−called koji mold, is very common, world-wide distributive and isolated from soil, cereals, foods and air.
Amano's *Aspergillus melleus* has been used safely for the production of food enzymes for over 35 years.

The production strain P-52 was obtained by several mutations of the original strain No.26st that was found Japanese soil. MSI (Mono Spore Isolation) and conventional mutagenesis using UV-irradiation, N-methyl-N'-nitrosoguanidine and ^{60}Co were used to obtain the current production strain P-52. The mutation history is provided in the Appendix D - 1. Recombinant DNA technology is not used to obtain this strain. It has been identified as A*spergillus melleus* by the third party, Japan Food Research Laboratories (Appendix D - 2).

IAM: Institute of Applied Microbiology, University of Tokyo Japan
The taxonomy of *Aspergillus melleus* is available at the NCBI taxonomy database (Taxonomy ID: 138277):

Super Kingdom	Eukaryote
Kingdom	Fungi
Phylum	Ascomycota
Order	Eurotiales Mucorales
Family	Trichocomaceae
Genus	Aspergillus
Species	Melleus

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

Aspergillus melleus that produces protease has been listed as safety microorganism (Pariza_Johnson). A literature search was performed on the PubMed Database in order to eventually identify academic works on the pathogenicity or toxinogenicity of the genus *Aspergillus* and *Aspergillus melleus*. This search was performed without any limit in time. Aside from being as common contaminants, this literature search showed that *Aspergillus* species occasionally cause infections in humans. However, zygomycetes infections are rare and these infections are mostly caused in immunosuppressed patients such as diabetics, leukemia, burn and those who have received an organ or stem cell transplant surgery (Kauffman, 2006). When the search was limited to *Aspergillus melleus* only, there was no reported *Aspergillus melleus* pathogenicity in human.

To demonstrate that the production strain does not produce toxicologically significant amounts of mycotoxins, we tested for the presence of mycotoxins such as Aflatoxin B1, Ochratoxin A, Sterigmatocystin, Zearalenone and T-2 Toxin. The result is as follows:

Table D - 1: The results of Mycotoxins

Batch no	PZH-N67-006	PZH-N67-009	PZH-N67-011
Aflatoxin B1	ND	ND	ND
Aflatoxin B2	ND	ND	ND
Aflatoxin G1	ND	ND	ND
Aflatoxin G2	ND	ND	ND
Total Aflatoxin	ND	ND	ND
Ochratoxin A	ND	ND	ND
HT-2 Toxin (HT-2)	ND	ND	ND
T-2 Toxin (T2)	ND	ND	ND
Zearalenone (ZON)	ND	ND	ND
Sterigmatocystin	ND	ND	ND
Fumonisin FB1	ND	ND	ND
Fumonisin FB2	ND	ND	ND

ND: Not detected

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

The source micro-organism is neither genetically modified nor self-cloned. The production strain were established by a repeated mutation and screening process from the prior strain. (Mutagen used: UV-irradiation, N-methyl-N'-nitrosoguanidine, Mono spore isolate, $Cobalt^{60}$) (Appendix D - 3 and Appendix D - 4)

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

In brief, to ensure the genetic stability of the source organism, the production strain is fermented and is divided into a flask. They are kept at 2-10°C in a locked desiccator. When ready, a flask 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

In principle, the enzymatic conversion of protein with the help of Oryzin (Protease) can be used in the processing of all food raw materials which naturally contain protein. The food enzyme object of this dossier is typically used in the following food manufacturing processes:

- Baking
- Dairy processing
- Egg processing
- Meat and fish processing
- Protein processing
- Yeast processing
- Flavoring production

Food enzyme preparations are used by food manufacturers according to the *Quantum Satis* principle, which means that food manufacturers will typically fine-tune the enzyme dosage based on a dose range recommended by the enzyme supplier.
The Table F-1 provides recommended dose ranges in the various food processes:

Table F - 1: Recommended Dose Ranges

Application	Raw material (RM)	Recommended use levels (mg TOS/kg RM)
Baking	Flour	6-59
Dairy processing	Milk and milk derived proteinic ingredients	59-1186
Egg processing	Eggs	59-1186
Meat and fish processing	Meat and fish	59-593
Protein processing	Proteins from various origin	59-1186
Yeast processing	Yeast	10-1000
Flavoring production	Material of vegetable, animal or microbial origin	10-1000

Doses are expressed in Total Organic Solids (TOS).

F.2 Anticipated Residue Levels of Oryzin (Protease)

The recommended use levels of the enzyme Oryzin (Protease) are given in Table F-2, based on the raw materials used in the various food processes.

Table F - 2: The Recommended Use Levels

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food	Ratio RM/final food	Maximal level in final food (mg TOS/kg food)
Beverages	Flavoring production	Material of vegetable, animal or microbial origin	1000	Flavourings used in various beverages	0.02	**20**
Solid food	Baking	Flour	59	Cereals	0.71	**42**
	Dairy processing	Milk and milk derived proteinic ingredients	1186	Enzyme modified cheese or dairy ingredient used in e.g. Soupe, Snacks and Processed cheeses…	0.05	**59**
	Egg processing	Eggs	1186	Processed egg products used in e.g. prepared foods, sauces, dressings, mayonnaise, bread, pastries, cake, desserts…	0.02	**24**
	Meat and fish processing	Meat and fish	593	Meat and fish extract used in e.g. prepared foods, snack foods, sausage and other meat-derived foods…	0.05	**30**
	Protein processing	Proteins from various origin	1186	Protein hydrolysates used in e.g. prepared foods, snack foods, sausage and other meat-derived foods…	0.05	**59**
	Yeast processing	Yeast	1000	Yeasts extracts used in e.g. savoury snacks, food supplements	0.02	**20**
	Flavoring production	Material of vegetable, animal or microbial origin	1000	Flavourings used in various solid foods	0.02	**20**

F.3 Information on the Likely Level of Consumption of Oryzin (Protease)

As is outlined above, Oryzin (Protease) from *Aspergillus melleus* may be used in the manufacture of a wide variety of foods, food ingredients and beverages. Due to this wide variety of applications, the most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

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

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

For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, it is assumed that all the TOS will end up in the final product. In the case of alcohol distillation, however, it is assumed that nothing of the TOS will end up in the final product due to the distillation process. Therefore, this application is not mentioned in the Table listed in F.2.

The Total TMDI can be calculated on basis of the maximal values found in food and beverage (in the above cases Flavoring production for beverages, Dairy and Protein processing for solid foods), multiplied by the average consumption of food and beverage/kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	**TMDI in beverage (mg TOS/kg body weight/day)**	**Total TMDI (mg TOS/kg body weight/day)**
20 x 0.0125 = 0.25	59 x 0.025 = 1.475	**1.725**

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

- It is assumed that ALL producers of the above mentioned foodstuffs and beverages use the specific enzyme Oryzin (Protease) from *Aspergillus melleus*;
- It is assumed that ALL producers apply the HIGHEST use level per application;
- For the calculation of the TMDI's in food as well as in beverage, only THOSE foodstuffs and beverages were selected containing the highest theoretical amount of TOS. Thus, foodstuffs and beverages containing lower theoretical amounts were not taken into account;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food containing the calculated theoretical amount of TOS is consumed DAILY over the course of a lifetime;

Assumptions regarding food and beverage intake of the general population are overestimates of the actual average levels (Douglass et al., 1997).

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

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

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

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

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

Not applicable.

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