Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include Thermolysin (Protease) from *Anoxybacillus caldiproteolyticus* as a Processing Aid

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Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include Thermolysin (Protease) from *Anoxybacillus caldiproteolyticus* as a Processing Aid

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Confidential Commercial Information (CCI)

Additional material is provided for CCI to support the application for Table A–1 of section A.4.2. The material is provided separately to this Application document.

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Petition to Amend Schedule 18 of the Australia New Zealand Food Standards Code to Include Thermolysin (Protease) from *Anoxybacillus caldiproteolyticus* as a Processing Aid

GENERAL REQUIREMENTS

1.0 APPLICANT DETAILS

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

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 thermolysin (protease) from *Anoxybacillus caldiproteolyticus* (EC 3.4.24.27) as a processing aid.
Thermolysin is a thermo-stable neutral metallo-proteinase and is used as an enzyme for protein processing to improve physical properties in foods.

3.0 JUSTIFICATION FOR THE APPLICATION

3.1.1 Regulatory Impact Information

3.1.1.1 Cost and Benefit of the Proposed Change

Cost and Benefit to consumers:
The inclusion of thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* in the Australia New Zealand Food Standards Code as a processing aid will no cost or benefits to consumers associated with the inclusion of thermolysin in the Schedule 18. The use of thermolysin is one of a number of commercial methods available to improve physical properties of certain food products. The availability of a range of food products is the same, irrespective of the method employed to achieve the results.

Cost and benefit to Industry;
It will allow producers to add a thermolysin (protease) step to their production process. The use of thermolysin (protease) has a lot of benefit in food processing. It specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids into amino acids and small peptides. These food groups are specifically, but not exclusively, dairy processing, egg processing, meat and fish processing, protein processing, yeast processing and flavoring processing. The main effect of the use of thermolysin (protease) is to improve physical properties (foamability, emulsifying ability, heat stability, viscosity) and also improves organoleptic properties (taste and flavor) and nutritional properties (absorptivity, digestivity).

Cost and benefit to Government;
There will be no additional cost to the regulator if the processing aid is approved as the use of thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* will not impact the regulation of these food products since processing aids are machinery in nature and their use is voluntary.

3.1.1.2 Impact on International Trade

The approval of thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* 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 thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* under the proposed conditions of use as a processing aid in Australia and New Zealand, as presented in accordance with the information requirements listed in Section 3.3.2 (Processing Aids) of the Food Standards Australia New Zealand (FSANZ) Application Handbook (FSANZ, 2016). The data pertaining to the thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* 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 thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus*, 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 thermolysin (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 thermolysin (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, 2016), any evidence that the food industry generally or other specific companies have an interest, in, or support, the proposed changes to the Code is mandatory for applications to change the Food Standards Code. As discussed in Section C, the use of thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* has a history of use in France and other countries in Europe. It is expected that the introduction of thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* 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 thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* as a processing aid, to be the General Procedure (Subdivision D), Cost Category Level 1 (up to 350 hours). This is based on the fact that FSANZ had approved the similar food enzymes; Metalloproteinase from various microorganisms and Amano Enzyme has a long history of selling this enzyme preparation to food industries.

6.0 CONFIDENTIAL COMMERCIAL INFORMATION

This application does contain information that is confidential commercial information (CCI). This information is provided separately and clearly labeled as CCI.

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:

- This food enzyme, thermolysin (protease), complies with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO, 2006). (See also A.5.1)
- *Geobacillus caldoproteolyticus* is the former name of *Anoxybacillus caldiproteolyticus*[1]. Protease from *Geobacillus caldoproteolyticus* (*Anoxybacillus caldiproteolyticus*) is approved in France[2].
- Protease from *Geobacillus caldoproteolyticus* (*Anoxybacillus caldiproteolyticus*) is listed in IPA Database by CCFA[3].

[1] http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2016.4522/full#efs24522-bib-0021
[2] https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000271061&dateTexte=#LEGIARTI000023482902
[3] http://ipa.ccfa.cc/substance?task=detail&substance_id=725

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 Thermolysin (Protease)

Thermolysin (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

Thermolysin (Protease) is powdered enzyme and is an enzyme catalyzing the hydrolysis of peptide bonds. The general reaction scheme is:

$$-R_1CH-CO-NH-CR_2H- + H_2O \longleftrightarrow -R_1CH-COO^- + {}^+H_3N-CR_2H-$$

Amano Enzyme has prepared thermolysin (protease) enzyme preparation that is derived from *Anoxybacillus caldiproteolyticus* 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, thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus* would fall under the following classification within Schedule 18 (Processing Aids):

18-4 (5) Permitted enzymes of microbial origin

The maximum proposed level of thermolysin (Protease) to food products use is 0.24%.

A.2 Information on the Identity of the Processing Aid

IUBMB name:	Thermolysin[4]
Common name:	Protease
Systematic name:	Thermolysin
EC number:	3.4.24.27
CAS registration number:	9073-78-3
EINECS number:	232-973-4

The thermolysin (protease) preparation is produced by *Anoxybacillus caldiproteolyticus* TP-7. Strain TP-7 is not genetically modified organism but a chemically mutated production

[4] http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/24/27.html

strain derived from the original strain (See also section D.1). *Anoxybacillus caldiproteolyticus* has been used for many years for food or feedstuffs purposes or in the production of enzymes processing aids in France and other countries of Europe.

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

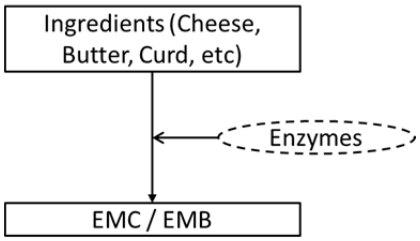
A.3.1 Purpose of using the processing aid

The use of thermolysin (protease) has a lot of benefit in food processing. It specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids into amino acids and small peptides. These food groups are specifically, but not exclusively, dairy processing, egg processing, meat and fish processing, protein processing, yeast processing and flavoring processing.

1) Dairy processing

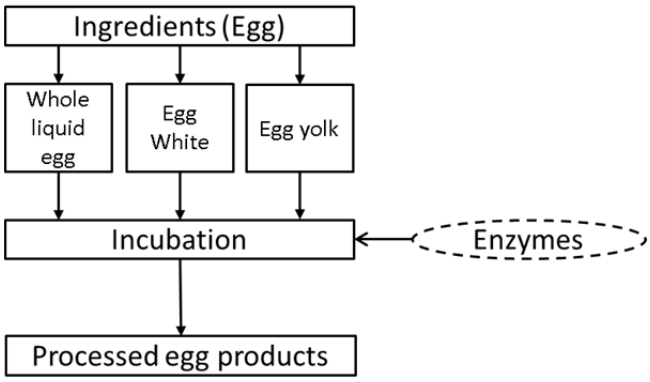
【Advantages of using thermolysin (protease)】

Thermolysin (Protease) can be used to produce EMC/EMB which is a high accelerated ripened cheese product. EMC/EMB can be used as a cheese/butter replacer in processed food products. Compositions of amino acids after using the enzyme are different depending on an enzyme. Therefore, these differences can be used to produce a variety of taste to enhance butter/cheese flavors.



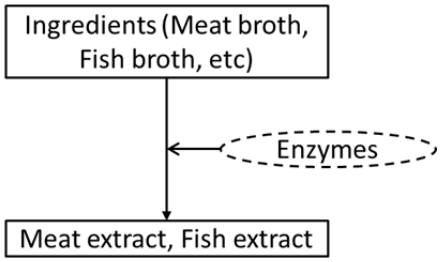
2) Egg processing

【Advantages of using thermolysin (protease)】
Thermolysin (Protease) can be used to strengthen egg flavor and improve its thermo tolerance. Using this enzyme can achieve to make eggs which are not congealed by heating.



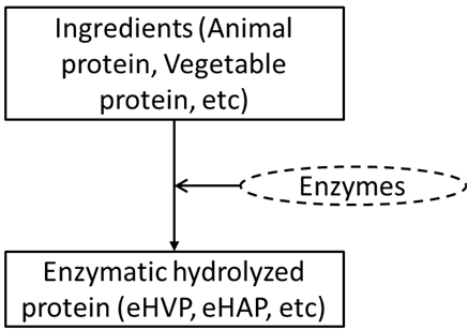
3) Meat and fish processing

【Advantages of using thermolysin (protease)】
Free amino acids and low molecular weight peptides, which are produced by using thermolysin (protease), can be used for improvements of taste, yield of extract, nutritional content, and physical properties.



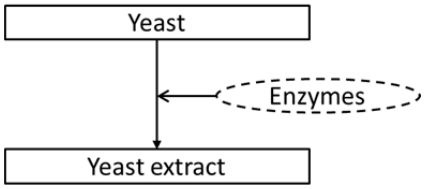
4) Protein processing

【Advantages of using thermolysin (protease)】
HVP and HAP, which are produced by using thermolysin (protease), are used for amino-acid-based seasoning. A variety of taste or flavor can be produced depending on the ingredients or reaction conditions.



5) Yeast processing

【Advantages of using thermolysin (protease)】
Flavor components of yeast extract are classified into a nucleic acid type, protein type and trace component type. The flavor components of a protein type are amino acids and peptides. Yeast extract produced by using thermolysin (protease) can be used for improvements of taste.



6) Flavoring processing

【Advantages of using thermolysin (protease)】
Enzymatic generation of flavor is widely used in the flavor industry. This enzyme, thermolysin (protease) also can be used for improvements of taste and flavor.

A.3.2 Technological Function and Enzymatic Properties

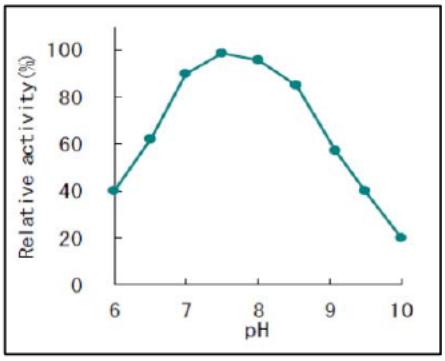
A.3.2.1 Assay for Measuring Thermolysin (Protease) Activity

An analytical method for the detection and quantification of thermolysin (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.3.2.2 Characterization of Thermolysin (Protease) Activity

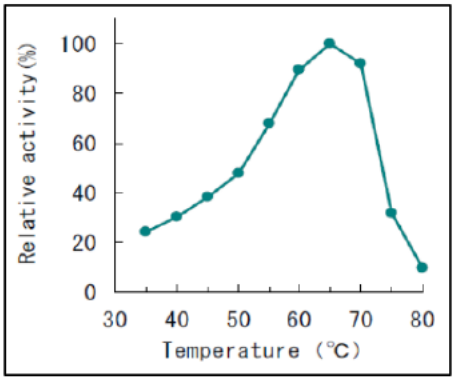
The technical function of thermolysin (protease) is to catalyze the hydrolysis of proteins broad specificity. The effects of temperature and pH on the activity of the thermolysin (protease) concentrate were examined and the results are presented in Figures A-1 and A-2. In all assays the same experimental procedures described above were employed with the only modifications affecting the temperature of the water bath or the pH of the thermolysin (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 thermolysin (protease) occurs at 60-70°C and a pH of approximately 7-8.5.

Figure A - 1: Effect of the pH



0.5% casein, Britton - Robinson buffer, 35°C, 10min incubation

Figure A - 2: Effect of temperature



0.5% casein, pH7.2, 10min incubation

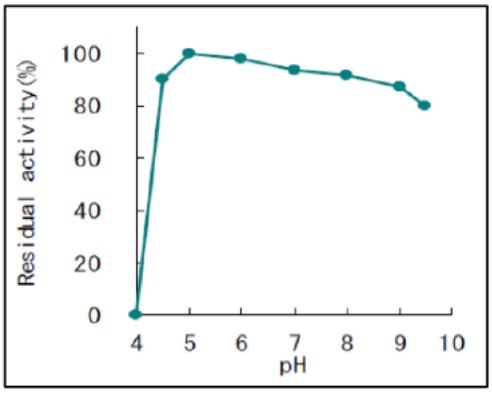
A.3.3 Stability

pH and THERMAL STABILITY

The stability of thermolysin (protease) has been assayed. As the enzyme activity was considered the primary marker of the stability of thermolysin (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.

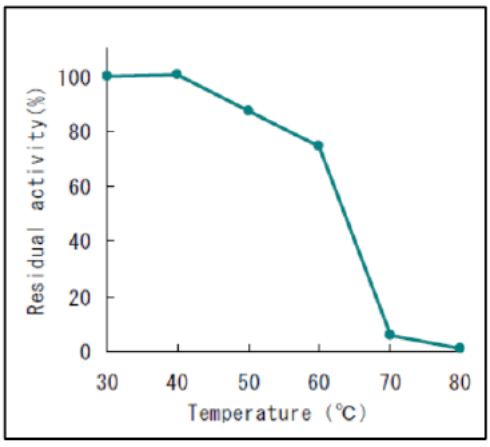
The results of the assessment of stability under varying temperature and pH conditions indicate that thermolysin (protease) is stable at 35-75°C and in a pH range of 6 to 10.

Figure A - 3: pH stability



60°C, 30min incubation

Figure A - 4: Thermal stability



1% Enzyme solution, 20mM phosphate buffer, pH7.0 60min

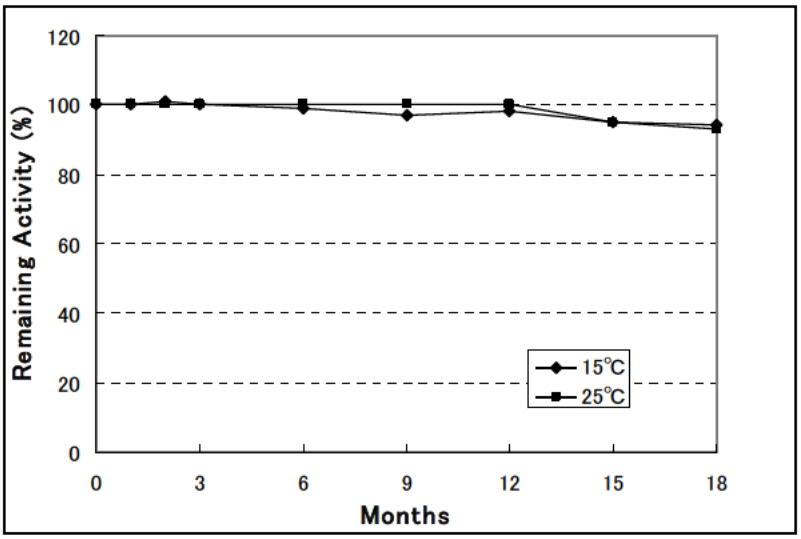
LONG TERM STABILITY

The stability of the enzyme preparation was assayed by the Amano Enzyme Inc. Samples were putted into an airtight bag and kept at 15°C and 25°C.

The protease activity was periodically measured for 18 months. Results are shown at the below figure.

It could be concluded that the protease activity remained over 90% of the initial activity after 18 months at both temperatures.

Figure A - 5: The stability of the enzyme preparation



A.3.4 Possible Interactions with Food Constituents

Thermolysin (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 increasing the temperature above 70°C. Food manufacturers conforming to the recommended conditions of use will ensure that the enzyme is inactivated in the final food product and therefore, unable to react with any protein present in non-target foods.

A.3.5 Characterisation of Secondary Activities

As far as Amano Enzyme is aware, the thermolysin (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.

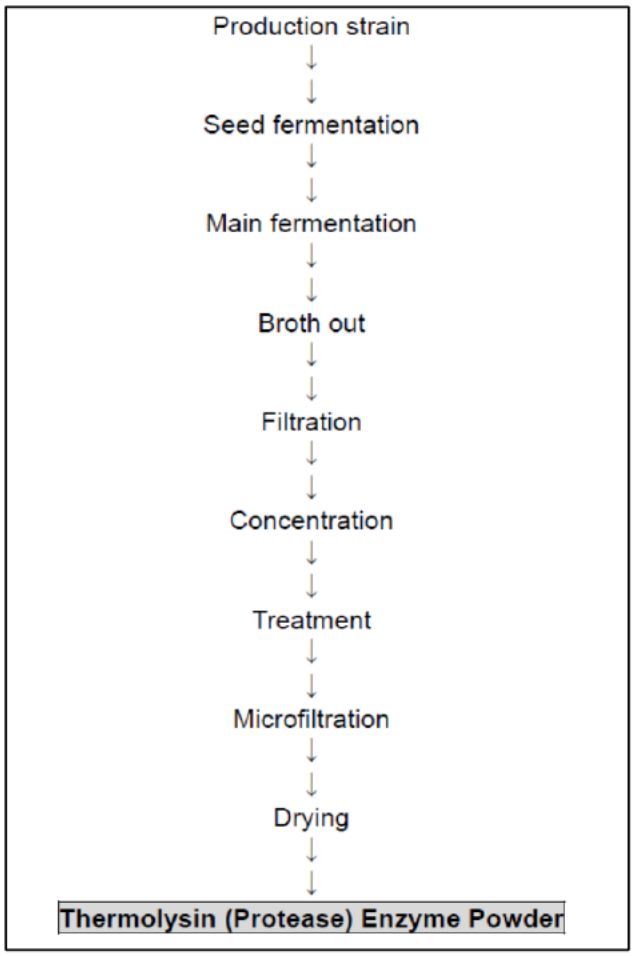
Finally proteases and phospholipases are natural constituents of foods. For instance, bromelain is a protease that is ingested in its active form by consumers eating raw pineapples. Phospholipase is a normal constituent of wheat flour (Nolte *et al.,* 1974) and is one of the digestive enzymes present 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 thermolysin (protease) is provided in Figure below.

Figure A - 6: Manufacturing Process



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

The enzyme preparation 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 thermolysin (protease) is listed in following table along with the grade of material employed, the function in the production process, and the status of the raw material in Australia and New Zealand. All of the raw materials employed in the production of thermolysin (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 for the production
(This table is considered as CCI and provided in the separate document.)

A.4.3 Residual Allergens from the Culture Medium

Soybean flour, milk casein and fish (Tuna) extract are used as fermentation media. Residual soy and milk protein were analyzed and resulted as “not more than the detection limit (1μg/g)” in thermolysin (protease) enzyme powder. As for the fish extract, only “salmon” and “mackerel” are designated as a specific food ingredient with potential to cause fish allergies in Japan. Therefore, analysis method to quantify residual tuna levels has not been established and validated so far.

It is described in the FSANZ’s “Review of the regulatory management of food allergens” that a number of fish allergens are temperature sensitive. Steam sterilization at 122±1^{o}C for 30min is carried out for fermentation media before the production strain is inoculated. It therefore can be expected that the fish allergens are denatured during this process.

Nevertheless, to make a numerical review, we calculated the highest-level (“worst-case”) estimate as follows;

・The fish extract used for the fermentation media is 13kg.
・Total volume of the fermentation media that excludes the water is 4,250kg.
・The fermentation media contain 0.3% fish extract. (13 / 4250×100)

Therefore, if we assume that all fermentation media remains in the thermolysin (protease) enzyme powder, thermolysin (protease) enzyme powder may contain up to 0.3% fish extract. And, as described in Section F.1, thermolysin (protease) is used at levels of up to 0.24%* to food products for enzyme reaction.
Fish allergen levels in food ingredients treated with thermolysin (protease) is therefore calculated to be; 0.3%×0.0024 = 7.2 ppm

Furthermore, food ingredients treated with thermolysin (protease) are applied to produce final food products consumed. Therefore, residual fish allergen level would be much lower level.
Based on the above, it can be concluded that the exposure to any potential allergens in final food products will be negligible and extremely unlikely to be of any allergenic concern.

*: Maximal use level to food product: 1,000 mgTOS/kg (See section F.1)
= 2,369mg/kg (TOS: 42.2%, see section A.5.2)
= 0.24 %

A.5 Specification for Identity and Purity

A.5.1 Product Specification

The Chemical and Microbiological Specification

It is proposed that the food enzyme thermolysin (protease) should comply with the internationally accepted JECFA specifications for chemical and microbiological purity of food enzymes (FAO/WHO[5]):

Table A - 2: Specification for Thermolysin (Protease)

The Chemical and Microbiological Specification as defined by JECFA	
Lead	Not more than 5 mg/kg
Salmonella spp.	Absent in 25 g of sample
Total coliforms	Not more than 30 per gram
Escherichia coli	Absent in 25 g of sample
Antimicrobial activity	Not detected
Mycotoxins	Not applicable for bacterial enzymes
General requirements as defined by Food Chemical Codex (FCC)	
Lead	Not more than 5 mg/kg
Coliforms	Not more than 30 cfu per gram
Salmonella	Negative in 25g
Enzyme Activity	
Protease activity	Not less than 90,000 u/g
General Properties	
Appearance	White powder

[5] http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/enzymes/en/

A.5.2 Batch Analysis

The proof that the food enzyme thermolysin (protease) complies with these specifications is shown by the analyses on various different batches, see Appendix A - 3.

Protein content and relative purity of the food enzyme thermolysin (protease) from *Anoxybacillus caldiproteolyticus* was measured, and the TOS values were calculated, in 3 batches. The result is shown in the following Table.

Table A - 3: Batch Analysis

Batch no	TP(SDD)-D64-001	TP(SDD)-D65-001	TP(SDD)-D65-002	Mean
Heavy metals				
Lead	0.007 mg/kg	0.060 mg/kg	0.005 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	Negative	Negative	Negative	-
Protein content and relative purity				
Ash (%)	54.8	56.0	55.1	55.3
Water (%)	2.7	2.5	2.3	2.5
TOS (%)	42.5	41.5	42.6	42.2
Enzyme activity	4,500,000	4,710,000	4,940,000	4,720,000
Units/mg TOS	10,588	11,349	11,596	11,178
Protein (%)	41.1	38.0	40.7	39.9

ND: Not detected

ABSENCE OF TOXINS

Anoxybacillus caldiproteolyticus (Former name: *Geobacillus caldoproteolyticus*) is not known to produce any bacterial toxins, which is why it is a common production organism for food processing enzymes. As the species *Anoxybacillus caldiproteolyticus* is a bacterium, it does not produce any mycotoxins.

A.6 Analytical Method for Detection

In accordance with Section 3.3.2 of the FSANZ Application Handbook, an analytical method for detection is not required for an enzymatic processing aid (FSANZ, 2016). Therefore, this section is not relevant to the use of thermolysin (protease) derived from *Anoxybacillus caldiproteolyticus.*

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

- *Geobacillus caldoproteolyticus* is the former name of *Anoxybacillus caldiproteolyticus*[6]. Protease from *Geobacillus caldoproteolyticus (Anoxybacillus caldiproteolyticus)* is approved in France[7].
- Protease from *Geobacillus caldoproteolyticus (Anoxybacillus caldiproteolyticus)* is listed in IPA Database by CCFA[8].

[6] http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2016.4522/full#efs24522-bib-0021
[7] https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000271061&dateTexte=#LEGIARTI000023482902
[8] http://ipa.ccfa.cc/substance?task=detail&substance_id=725

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

As mentioned in Section C.1, thermolysin (protease) enzyme preparations have a wide history of use in food processing. To further support the safety of thermolysin (protease) enzyme preparation, several toxicity studies have been conducted to assess the safety. The potential mutagenic and genotoxic activity of the thermolysin (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 food enzyme has been subjected to a standard package of toxicological tests, with the following results:

- Micronucleus: 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 1,000 mg/kg bw/day (323.3mg TOS/kg bw/day), which is the high dose in the study.

ABSENCE OF TOXINS

Anoxybacillus caldiproteolyticus (Former name: *Geobacillus caldoproteolyticus*) is not known to produce any bacterial toxins, which is why it is a common production organism for food processing enzymes. As the species *Anoxybacillus caldiproteolyticus* is a bacterium, it does not produce any mycotoxins.

C.2.1 Thermolysin (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.

It is known that a set of the reverse mutation test in bacteria and the chromosomal aberration study is normally used to evaluate enzymes safety. However, micronucleus test was conducted for this enzyme instead of the reverse mutation test. The reason of why the micronucleus test was conducted is that it is known about the possibility that the result of the reverse mutation test using protease would be positive because the increase of the revertant colonies might be attributable to histidine contained in protease. Therefore, the micronucleus test and the chromosomal aberration study were conducted for this enzyme.

Micronucleus Test in rat (Appendix C - 1)

Thermolysin was administered orally to mice, and any mutagenicity due to the induction of abnormal gene formation indicated by the micronucleus formation was examined.

There was no difference in the appearance ration of the micronucleus in the thermolysin administered group (1,250 to 5,000 mg/kg, 1 dose or 4 doses, and sample collected after 24 to 72 hours) from the control group (only the solvent was administered).

Ratio of the polychromatic erythrocytes in the whole erythrocytes in the thermolysin groups was not different from that in the control group.

Thus, it may be concluded that thermolysin has no mutagenicity due to the induction of micronucleus.

Chromosomal Aberration Test (Appendix C - 2)

A chromosome aberration study was conducted using cultured Chinese hamster lung fibroblast (CHL/IU) cells to examine whether thermolysin has the potential to induce chromosome aberrations.

Based on the results of a cell-growth inhibition test which was conducted to determine dose concentrations, a chromosome aberration test was conducted by providing a total of 6 dose concentrations diluted with a common ratio of 1.2 including the highest dose concentration of 80.0μg/mL in the short-term and the continuous-term treatment methods.

In the short-term treatment and continuous treatment (retest), the incidence of abnormal cells excluding gap (TA value) and the incidence of polyploid cells were less than 5% at all dose concentrations. Since the negative control group and positive control group showed the TA value within the background data of the test facility and the occurrence of polyploid

cells in all treatments, it was considered that the study was conducted appropriately. Based on the above results, it was concluded that thermolysin had no potential to induce chromosome aberrations under the conditions of this study.

C.2.1.2 Repeat Dose Toxicity Assay

Sub-chronic toxicity (13-week oral toxicity study) (Appendix C - 3)

Reference :

Tyerman Y. (1989) 91 day oral gavage toxicity study with THERMOASE (RCC NOTOX Substance 2537) in the rat. RCC NOTOX B. V., Hambakenwetering, NL. Study No. RCC NOTOX 008696. 373 pages.

GLPs and QA:

The study was performed under quality assurance and complies with Good Laboratory Practices (GLPs).

Summary:

A 91 days subchronic oral toxicity study (by gavage) was performed in 1989 by the RCC NOTOX B. V. (Hambakenwetering, Netherlands).
The study was performed according to the US-FDA guidelines: toxicological principles for the safety assessment of direct food additives and colour additives used in food (US Food and Drug Administration, Bureau of Foods1982).
The thermolysin (lot n° 1006 SD1) was administered daily by gavage at doses of 0, 40, 200 and 1,000 mg/kg bodyweight per day. Each group was constituted of 20 males and 20 females (6 week old). The vehicle was 1% methyl cellulose.
At the end of the administration period, the following parameters were measured: general conditions, body weights, food consumption, ophthalmology, hematology, blood chemistry, pathology and histopathology.
During the test period, no deaths and no clinical signs were observed. No toxicological relevant protease-related changes in body weight, food consumption, ophthalmology, hematology, blood parameters, blood chemistry, gross pathology and histopathology were detected.
Males receiving 1,000 mg/kg/day had slight, but statistically significant decreased red blood cell numbers, decreased haematocrit values, decreased platelets and decreased numbers of large unstained cells when compared to controls. These effects were not considered as toxicologically relevant because no changes were observed on hematopoietic organs.
Minor blood chemistry changes were observed in males and females. These changes were not dose-related and not considered to be of toxicological significance.
Some incidental macroscopic findings were noticed: reduction in testis size, red discolouration of the urinary bladder, haemorrhage of the thymus, enlarged lymph nodes, pelvic dilation of the kidneys, reduction in size of the epididymides, red discolouration to the

spinal cord, brown discolouration of the lung, red discolouration of the mandibular lymph nodes and swollen uterus, relative adrenal weights. These changes were not considered treatment-related because being within the range of values normally expected for rats of this age and strain.

In conclusion, no treatment-related changes were found in the tested animals, except some minor changes which were not considered toxicologically relevant.

The no-effect level of protease in this sub-chronic study was thus considered to be 5.0% (w/w) corresponding to 1,000 mg/kg/day for both males and females.

(CALCULATION of TOS)

At the time of systemic toxicity study, the total organic solid of the test article was not determined. Three samples of concentrate were collected in 2011 and analyzed for water and ash contents. Considering that the process of production didn't change, this allows determining a mean TOS of 32.33% as indicated in table below.

Test Items	Lot No.		
	TP1001223.00CP	TP-D63-001	TP-D63-002
Water (%)	3.87	2.61	2.94
Ash (%)	64.1	62.4	67.1
TOS (%)	32.03	34.99	29.96
Mean TOS (%)	**32.33**		

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 1.53 mg TOS/kg body weight/day. Consequently, the MoS is:

$$MoS = 323.3 / 1.53 = 211$$

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

Geobacillus caldoproteolyticus (Anoxybacillus caldiproteolyticus) has been approved as a processing aid in France and has been used in Europe. No allergenicity warnings are associated with the use of this organism in foods. Also, no adverse effects have been reported in workers exposed to *Geobacillus caldoproteolyticus (Anoxybacillus caldiproteolyticus)*.

C.3.2 Allergenicity of Thermolysin (Protease)

Amino-acid sequence

The amino-acid sequence for the thermolysin (protease) from *Anoxybacillus caldiproteolyticus* enzyme protein has been determined as indicated below.

```
1   MKRKMKMKLR SFGVAAGLAA QVFLPYNRLA SSEHVTWNQQ FQTPQFISGD LLKVNGTSPE   60
61  ELVYQYVEKN ENKFKFHENA KDTLQLKEKK NDNLGFTFMR FQQTYKGIPV FGQVVTAHVK  120
121 DGSLTALSGT LIPIPNLDTK GSLKSGKKLS EKQARDIAEK DLVANVTKEV PEYEQGKDTE  180
181 FVVYVNGDEA SLAYVVNLNF LTPEPGNWLY IIDAVDGKIL NKFNQLDAAK PGDVKSITGT  240
241 STVGVGRGVL GDQKNINTTY SSYYYLQDNT RGNGIFTYDA KYRTTLPGSL WADADNQFFA  300
301 SYDAPAVDAH YYAGVTYDYY KNVHNRLSYD GNNAAIRSSV HYSQGYNNAF WNGSQMVYGD  360
361 GDGQTFIPLS GGIDVVAHEL THAVTDYTAG LIYQNESGAI NEAISDIFGT LVEFYANKNP  420
421 DWEIGEDVYT PGISGDSLRS MSDPAKYGDP DHYSKRYTGT QDNAGVHINS GIINKAAYLI  480
481 SQGGTHYGVS VVGIGRDKLG KIFYRALTQY LTPTSNFSQL RAAAVQSATD LYGSTSQEVA  540
541 SVKQAFDAVG VK                                                     552
```

The homology search of the EFSA CEF Guidance document on food enzymes (EFSA, 2009b) could be performed.

The result shown that only one match of a known allergen was observed in the matching for 8- consecutive amino acid sequence search. However this match was thought to be accidental and single match of continuous amino acids observed between a novel protein and an allergen may not be clinically relevant (see Appendix C - 4).

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

Since 1980, Amano have sold it for food use. No adverse effects have been reported in workers exposed either to the parent strain or to the enzyme preparation.

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

There is no safety assessment report that can be provided.

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 *Anoxybacillus caldiproteolyticus*. The wild type strain, *A. caldiproteolyticus*, is very common and widely distributed in soil, hot springs and ocean sediment.

- Amano's *Anoxybacillus caldiproteolyticus* has been used safely for the production of food enzymes for many years.

- The production strain TP-7 was obtained by several mutations of the original strain that was found Japanese soil. The production strain is derived via selection by conventional mutagenesis using NTG (N-methyl-N'-nitro-N-nitrosoguanidine) (Appendix D - 1).

- Recombinant DNA technology is not used to obtain this strain. It has been identified as *Anoxybacillus caldiproteolyticus*.

- The taxonomy of *Anoxybacillus caldiproteolyticus* is available at the NCBI taxonomy database (Taxonomy ID: 247480[9]):

Genus	*Anoxybacillus*
Species	*caldiproteolyticus*
Synomims	*Geobacillus caldoproteolyticus*

- Identification test of the production strain (TP-7) was performed and was identified as *Anoxybacillus caldiproteolyticus* (Appendix D - 2).

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

International safety classification

With reference to the Risk Classification of the European Community (Directive 2000/54/EC[10]) *Anoxybacillus caldiproteolyticus* could be classified in Group 1: biological agent that is most unlikely to cause human disease. And according to risk classifications of other organisations such as DSMZ (Deutsche Sammlung von Mikroorganismen und

[9] https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi
[10] http://www.biosafety.be/PDF/2000_54.pdf

Zellkulturen GmbH) and ATCC (the American Type Culture Collection), this strain is classified as a safe agent (risk group 1 or biosafety level 1) (Appendix D - 3 and Appendix D - 4). Although the appendix D – 4 refers to *Geobacillus caldoproteolyticus*, it is known that *Geobacillus caldoproteolyticus* is the former name of *Anoxybacillus caldiproteolyticus*[11].

Secondary metabolites

Concern on possible involvement of Bacillus cereus-like enterotoxins in the rare cases where some Bacillus strains have been associated with food poisoning caused the Scientific Committee on Animal Nutrition to require specific testing of industrially used Bacillus strains . The current view is that the very few reports of Bacillus cereus-like enterotoxins occurring in other species of Bacillus and related genera are likely to have resulted from a misidentification of the strain involved (EFSA Journal 2011;[9](11):2445)

In 2011 EFSA updated the guidance contained in the SCAN opinion stating that it now seems unlikely that Bacillus cereus-like enterotoxins are produced in species other than the Bacillus cereus group, and any toxigenic potential in other species appears far more likely to arise from the production of surfactins (EFSA, 2011[12]). A PCR detection of non-ribosomal peptide synthase genes is suggested to be adequate to identify surfactin-positive strains. However, among the 22 publically available full genome sequences of Bacilli of the Bacillus subtilis cluster the frequency of genes encoding for lipopeptide production is 100%, indicating that the presence of such genes are widespread, and this would not be a valid test.

EFSA recently updated the guidance document for the assessment of toxigenic potential of the Bacillus species (EFSA, 2014[13]). In this new Guidance, EFSA acknowledges that the relation between the presence of surfactin like-lipopeptides and/or other toxic factors and the risk of illness in human has not been established. Therefore, the test for the presence of non-ribosomal peptide synthase genes is no longer a requirement. Concerns related to haemolysis as a proof of cytotoxicity of Bacilli led EFSA to also remove this requirement. Although EFSA recognises the inherent uncertainty of the use of cytotoxicity assays an in vitro study with culture supernatant is still required as at present more reliable alternative methods are not available. The validated cytotoxicity studies performed by AMFEP members on representative Bacillus production strains confirm that, in the absence of any further concerns, the safety and quality management systems of the enzyme industry are

[11] http://onlinel brary.wiley.com/doi/10.2903/j.efsa.2016.4522/full#efs24522-bib-0021
[12] http://www.efsa.europa.eu/en/efsajournal/doc/2445.pdf
[13] http://www.efsa.europa.eu/de/supporting/doc/587e.pdf

adequate safeguards for the use of enzymes deriving from *Bacillus* (*amyloliquefaciens /subtilis*; FEFANA and AMFEP 2013).

Metabolites of human toxicological concern are usually produced by microorganisms for their own protection. Microbes in natural environments are affected by several and highly variable abiotic (e.g. availability of nutrients, temperature and moisture) and biotic factors (e.g. competitors and predators). Their ever changing environments put a constant pressure on microbes as they are prompted by various environmental signals of different amplitude over time. In the wild this results in continuous adaptation of the microbes through inducing different biochemical systems; e.g. adjusting metabolic activity to current availability of nutrients and carbon source(s), or activation of stress or defense mechanisms to produce secondary metabolites as 'counter stimuli' to external signals (Klein and Paschke, 2004, Earl et al., 2008). On the contrary, 'environmental' conditions of microbial production strains during industrial scale fermentation have been optimized and 'customized' to the biological requirements of the strain in question (Sanchez and Demain, 2002). Thus, the metabolic activity and growth of a particular microbial production strain during the fermentation process (primarily the 'exponential growth phase') will focus on efficiently building cell biomass which in turn produces the molecule of interest. Industrial fermentations are run as monocultures (i.e. no external competitors or predators) with optimal abiotic conditions. Hence, there are no strong environmental signals that would induce stress (e.g. starvation, competitive environment or low/high temperature) or defence mechanisms (e.g. production of antibiotic, antiviral or neurotoxic molecules). Biosynthesis of stress and/or defence secondary metabolites of toxicological relevance by industrial microbial production organisms during the fermentation process is thus highly unexpected (Sanchez and Demain, 2002) and is furthermore avoided from an economical perspective to optimize enzyme production.

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 process from the prior strain. (Mutagen used: N-methyl-N'-nitro-N-nitrosoguanidine) (Appendix D - 5 and Appendix D - 6)

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).

To ensure the genetic stability of the source organism, the production strain is fermented and is divided into an ampule. They are kept at below -70°C in a locked freezer.
When ready, an ampule is used for 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 order to confirm that the strain of the source organism does not undergo strain drift and that the culture conditions can be applied consistently between batches, enzyme activities and pH of the broth obtained after completing the fermentation are confirmed.
In any instance where a deviation from normal is detected in either of these parameters, the fermentation media is removed from production and discarded. The strain is then checked to ensure that no genetic drift has occurred.

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

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

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

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

F.1 Proposed Food Uses

The technical function of thermolysin (protease) is to the hydrolysis of peptide bonds containing hydrophobic amino acids into amino acids and small peptides.
Thermolysin (Protease) will be used in variety of food manufacturing processes including:

- 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 below provides recommended dose ranges in the various food processes:

Table F - 1: Recommended Dose Ranges

Application	Raw material (RM)		Recommended use levels (mg TOS/kg RM)
Dairy processing	Milk and milk derived proteinic ingredients		13-63
Egg processing	Eggs		13-101
Meat and fish processing	Meat and fish	Extract	13-63
		Softening	4-82
Protein processing	Proteins from various origin		13-63
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 Thermolysin (Protease)

The recommended use levels of the enzyme thermolysin (protease) are given, based on the raw materials used in the various food processes.

Table F - 2: 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	Dairy processing	Milk and milk derived proteinic ingredients		63	Enzyme modified cheese or dairy ingredient used in e.g. Soupe, Snacks and Processed cheeses…	0.05	**3**
	Egg processing	Eggs		101	Processed egg products used in e.g. prepared foods, sauces, dressings, mayonnaise, bread, pastries, cake, desserts…	0.2	**20**
	Meat and fish processing	Meat and fish	Extract	63	Meat and fish extract used in e.g. prepared foods, snack foods, sausage and other meat-derived foods…	0.05	**3**
			Softening	82	Grilled product, stewed product, Fried product and Braised/Boiled product	1	**82**
	Protein processing	Proteins from various origin		63	Protein hydrolysates used in e.g. prepared foods, snack foods, sausage and other meat-derived foods…	0.05	**3**
	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 Thermolysin (Protease)

As is outlined above, thermolysin (protease) from *Anoxybacillus caldiproteolyticus* 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)
82 x 0.0125 = 1.03	20 x 0.025 = 0.50	1.53

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 thermolysin (protease) from *Anoxybacillus caldiproteolyticus*;
- 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

The enzyme does not have any function (activity) in final foods since the enzyme is only used as a processing aid and is inactivated or removed during food production process. Maximal level described in the section F.2 is the level when we assume if all enzymes remain in final foods.

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

Not applicable.

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