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Supporting document 1

Risk and technical assessment – Application A1288

Thermolysin from *Anoxybacillus caldiproteolyticus* Rokko as a processing aid

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Executive summary

IFF Australia Pty Ltd (trading as Danisco Australia Pty Ltd) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme thermolysin (EC 3.4.24.27) from the Rokko strain of *Anoxybacillus caldiproteolyticus* as a processing aid for protein hydrolysis in the manufacture and/or processing of dairy foods, eggs, meat and fish, protein concentrates and isolates, yeast and in beer brewing.

The available evidence provides adequate assurance that the proposed use of thermolysin from *A. caldiproteolyticus* Rokko as a processing aid is technologically justified. Thermolysin performs its primary technological function during food processing and, as such, meets the definition of a processing aid. The enzyme preparation meets international purity specifications.

The microbiological assessment undertaken by FSANZ did not identify any public health and safety concerns associated with the use of *A. caldiproteolyticus* Rokko as a source of thermolysin.

The enzyme has been used in some countries overseas for more than 10 years. No adverse effects have been reported. Bioinformatics analysis found no significant homology of the enzyme with known toxins or food allergens. Glucose and sorbitol (sourced from wheat), and soy meal could be used as fermentation nutrients in the manufacture of thermolysin.

Thermolysin was not genotoxic *in vitro* and did not cause adverse effects in a 13-week oral toxicity study in rats. The no observed adverse effect level (NOAEL) in this study was 337.5 mg total organic solids (TOS)/kg bw/day, the highest dose tested.

The theoretical maximum daily intake (TMDI) of the TOS from the enzyme preparation was calculated to be 2.45 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 100.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard, an acceptable daily intake (ADI) ‘not specified’ is appropriate.

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1 Introduction

IFF Australia Pty Ltd (trading as Danisco Australia Pty Ltd) applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme thermolysin (EC 3.4.24.27) from the Rokko strain of *Anoxybacillus caldiproteolyticus* as a processing aid.

The enzyme may be used to hydrolyse peptide bonds during the manufacture and/or processing of protein-containing foods – dairy foods, eggs, meat and fish, protein concentrates and isolates, yeast and in beer brewing. It will be used at the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Practice (GMP).[1]

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is solely a technological function and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety concerns that may arise from the use of this enzyme as a processing aid by considering the:
  - safety and history of use of the production microorganism
  - safety of the enzyme preparation.

2 Food technology assessment

2.1 Identity of the enzyme

The applicant provided information regarding the identity of the enzyme and this has been verified using the IUBMB[2] enzyme nomenclature reference database (McDonald and Tipton 2023). Details of the identity of the enzyme are provided below.

Accepted IUBMB name:	Thermolysin
Other names/common names:	*Bacillus thermoproteolyticus* neutral proteinase, thermoase, thermoase Y10, TLN
IUBMB enzyme nomenclature:	EC 3.4.24.27
CAS number:	9073-78-3
Reaction:	Hydrolysis of proteins, with preference for cleavage of bonds in which Leucine (Leu) and Phenylalanine (Phe) are involved.

[1] GMP is defined in section 1.1.2—2 of the Code as follows: ***GMP*** *or* ***Good Manufacturing Practice,*** *with respect to the addition of substances used as food additives and substances used as processing aids to food, means the practice of:*
(a) limiting the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect; and
(b) to the extent reasonably possible, reducing the amount of the substance or its derivatives that:
*(i) remains as a *component of the food as a result of its use in the manufacture, processing or packaging; and*
(ii) is not intended to accomplish any physical or other technical effect in the food itself.
(c) preparing and handling the substance in the same way as a food ingredient.

[2] International Union of Biochemistry and Molecular Biology.

2.2 Manufacturing process

2.2.1 Production of the enzyme

Enzymes produced from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. Final standardisation with stabilisers, preservatives, carriers, diluents, and other approved food-grade additives and ingredients is carried out after the purification and concentration steps.

The formulated enzymes are referred to as enzyme preparations, which, depending upon the application in food, may be a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction during food processing or two or more active enzymes that catalyse different reactions (FAO/WHO 2020b).

Thermolysin is produced by submerged fed-batch pure culture fermentation of *A. caldiproteolyticus* Rokko. The fermentation process comprises three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

The recovery process involves multiple steps to separate the *A. caldiproteolyticus* Rokko biomass from the enzyme-containing culture medium, and then purification, concentration, and stabilisation of the thermolysin enzyme. This is accomplished through filtration or centrifugation of the fermentation broth, followed by ultrafiltration to eliminate low molecular weight compounds. Diafiltration is used to achieve the target enzyme activity and remove colour and smaller substances.

The applicant stated that enzyme production is conducted in accordance with GMP and the resultant product meets the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO 2005) and the Food Chemicals Codex (FCC) (13th edition) (FCC 2022).

Information on the raw materials and formulation ingredients used in fermentation and recovery is confidential commercial information (CCI). FSANZ has considered this information as part of this assessment but has not included it in this report because it is protected from disclosure under the Act.

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food, established by JECFA in its Compendium of Food Additive Specifications (FAO/WHO 2005) and in the FCC (13th edition) (FCC 2022), referenced in section 3—2 of Schedule 3 of the Code. Enzymes used as processing aids need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3.

Schedule 3 of the Code includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided the results of analysis of three different batches of the thermolysin preparation. Table 1 provides a comparison of the summary results of those analyses with international specifications established by JECFA and the FCC, as well as those in the Code. Based on those results, the enzyme met all relevant specifications.

In addition, the specification for identity and purity of the enzyme preparation (as stated by

the applicant in section 2.5 of the application) indicates an absence of the production strain. This is supported by the certificate of analysis of the test material provided by the applicant as CCI information.

Table 1: Analysis of applicant's liquid enzyme preparation compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Test parameters	Test results	Specifications		
		JECFA	Food Chemicals Codex	The Code – section S3—4
Lead (mg/kg)	<0.5	≤5	≤5	≤2
Arsenic (mg/kg)	<0.5	-	-	≤1
Cadmium (mg/kg)	<0.1	-	-	≤1
Mercury (mg/kg)	<0.1	-	-	≤1
Coliforms (cfu/g)	<1	≤30	≤30	-
***Salmonella* (in 25 g)**	Absent	Absent	Negative	-
***Escherichia coli* (in 25 g)**	Absent	Absent	-	-
Antimicrobial activity	Negative by test	Absent	-	-

cfu = colony forming units

2.3 Technological purpose

Thermolysin is a thermostable extracellular metalloendopeptidase. It hydrolyses proteins with preferential cleavage of bonds which contain Leucine (Leu) and Phenylalanine (Phe). The main reaction products are protein fragments of various lengths, peptides and free amino acids.

The applicant stated thermolysin is effective in raw material processing applications where a high level of thermostability is desirable. It has broad substrate specificity, and efficiently hydrolyses casein, whey proteins, gelatin, soy proteins, wheat gluten, fish proteins and other proteins.

The applicant stated the benefits of using the enzyme include flavour improvement, improved physical properties, increased yields (e.g. extracts), and processing efficiencies. The enzyme preparation is used at the minimum level required to achieve the desired effect, in accordance with the principles of GMP.

The highest recommended use level is 163 mg total organic solids (TOS) per kilogram of raw material.

Thermolysin performs its primary technological function during food processing and, as such, meets the definition of a processing aid. Information on the physical and chemical properties of the enzyme preparation is summarised in Table 2.

Table 2: Thermolysin enzyme preparation physical/chemical properties

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	Dependent on final product (measured in Thermolysin activity units (U/g))
Appearance	Brown liquid
Temperature range	Optimum activity within range 60 - 70°C
Thermal stability	No enzyme activity detected after 10 minutes at temperatures above 90°C
pH range and optimum	Optimum activity within range 5 - 6

2.4 Allergen considerations

The applicant has advised that glucose and sorbitol (sourced from wheat), and soy meal could be used as fermentation nutrients in the manufacture of thermolysin.

Hypothetical 'worst case' exposures to wheat and soy allergens via the enzyme preparation were estimated (Appendix G of the application). For glucose and sorbitol, using a worst-case scenario of 10 ppm total wheat protein in sorbitol or glucose, IFF determined that a level of 5 ppb wheat protein may be present in the final food. For soy, assuming that all of the soy protein present in the fermentation media passes unaltered to the finished food via the enzyme preparation, IFF estimated an upper limit of soy protein in the final food in the range of 1.1 mg per 100 g.

Depending on the specific product formulation, sorbitol may also be added as an ingredient to the enzyme preparation.

2.5 Food technology conclusion

The use of thermolysin as a processing aid for protein hydrolysis in the manufacture and/or processing of dairy foods, eggs, meat and fish, protein concentrates and isolates, yeast and in beer brewing is consistent with its functions and the functions of proteases more generally. Its stated benefits include flavour improvement, improved physical properties, increased yields (e.g. extracts), and processing efficiencies. The evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified.

Thermolysin performs its technological purpose during the manufacture of food products. It will be inactivated as a result of downstream protein processing, and so is not performing a technological purpose in the final food. It is therefore functioning as a processing aid for the purposes of the Code.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant has provided evidence that the enzyme meets these specifications.

3 Hazard assessment

3.1 Source microorganism assessment

3.1.1 Host organism

The taxonomic identity of the production organism has changed multiple times since 1989.

The applicant stated that when they acquired the organism it was defined as *Bacillus stearothermophilus var. thermoproteolyticus.* In 2001, *B. stearothermophilus* and its variant strains were transferred to the new genus *Geobacillus* (Nazina et al. 2001). Following this, the organism was reclassified as a novel species termed *Geobacillus caldoproteolyticus* (Chen et al. 2004). In 2012, *Geobacillus caldoproteolyticus* was reclassified again as *A. caldiproteolyticus* (Coorevits et al. 2012). The most recent nomenclature change was proposed by Patel et al. (2024). The proposed new name, *Thermaerobacillus caldiproteolyticus,* is validly published under the International Code of Nomenclature of Prokaryotes (Parte et al. 2020). The nomenclature change was brought to the applicant's attention and they chose to continue the assessment under the homotypic synonym *A. caldiproteolyticus.* This decision also allows FSANZ to maintain nomenclature consistency within the Code.

A. caldiproteolyticus is a gram positive, thermophilic, spore-forming bacteria that can be isolated from a range of environments including soil, hot-springs and deep sea sediments (Cheng et al. 2021, Zeigler 2014). *A. caldiproteolyticus* is a source of various enzymes of commercial interest such as cyclomaltodextrin glucanotransferase, alpha-amylase and thermolysin and has been used globally as an enzyme producer for decades (Codex 2024).

A. caldiproteolyticus is non-pathogenic and is classified as a biosafety level 1 organism (ATCC, Ito 1981, Turnbull 1996). However, the European Food Safety Authority (EFSA 2016) did not recommend *A. caldiproteolyticus* for Qualified Presumption of Safety (QPS) status due to the lack of a sufficient body of knowledge on a safe history of use or presence of the organism in foods and feeds. As an enzyme producer, *A. caldiproteolyticus* has been assessed by JECFA and EFSA and approved for use in many countries which regulate the use of food enzymes, such as Australia, the USA and Canada (Codex 2024, EFSA 2024, US FDA 2025, Health Canada 2025). FSANZ has previously approved *A. caldiproteolyticus* for the production of thermolysin as part of Application A1146 (FSANZ 2018). FSANZ notes the specific strain used in this application (Rokko) is different to the previously assessed strain (TP-7).

The applicant provided data that adequately demonstrates the production strain's identity as *A. caldiproteolyticus.* The production organism is removed from the fermentation broth through filtration and/or centrifugation. Microbiological testing of three independent batches was provided to FSANZ confirming the absence of the production organism in the final enzyme preparation.

The microbiological assessment undertaken by FSANZ did not identify any public health and safety concerns associated with the use of *A. caldiproteolyticus* as a source of thermolysin.

3.2 Safety of the thermolysin enzyme

3.2.1 History of safe use

In 2018 FSANZ approved the use of a thermolysin enzyme sourced from *Anoxybacillus caldiproteolyticus* strain TP-7 for use as a processing aid under Application A1146.

The thermolysin from *A. caldiproteolyticus* Rokko that is the subject of the present application has been used for protein hydrolysis in food in multiple countries including France (since 2007), Denmark (since 2009) and China (since 2021). The applicant has stated that there have not been any adverse events reported since thermolysin has been in commercial use in these countries.

3.2.2 Bioinformatic assessment of homology with known toxins

Results of a recent (2023) bioinformatics search for similarity of the amino acid sequence of thermolysin from *A. caldiproteolyticus* Rokko to those of known protein toxins and antinutrients were submitted as part of the application. The search was performed using the UniProtKB database (UniProt)[3]. No biologically relevant similarity with known toxins was found.

3.2.3 Toxicology data

The applicant submitted several proprietary toxicological studies conducted with their thermolysin enzyme preparation which were reviewed in the present assessment:

- Bacterial reverse mutation assay
- *In vitro* mammalian cell chromosomal aberration test
- 90-day oral toxicity study in rats

Genotoxicity studies

Two genotoxicity studies with the applicant's thermolysin preparation were submitted. These studies were conducted in accordance with GLP and OECD Test Guidelines. The positive controls in these studies produced the expected responses. The results of these studies, as summarised in Table 3, showed no evidence of mutagenicity or clastogenicity.

Table 3: Genotoxicity studies of thermolysin from *A. caldiproteolyticus* Rokko

Test[1]	Test object	Concentration	Purity (% total organic solids)	Results
Bacterial reverse mutation test (OECD TG 471 [1997])	*Salmonella typhimurium* strains TA98, TA100, TA1535 & TA1537; *Escherichia coli* strain WP2 uvrA pKM101[2]	With S9: 67 – 1075 µg/mL Without S9: 4 – 67 µg/mL (TA98 and TA1535); 17 – 538 µg/mL (TA100); 2 – 34 µg/mL (TA1537); 67 – 1075 µg/mL (*E. coli* WP2 urvA)[3]	33.75%	Negative ± S9[4]
Chromosome aberration test in vitro (OECD TG 473 [1997])	Human peripheral blood lymphocytes[5]	Experiment I: 1.68 – 215 µg/mL Experiment II: 6.72 – 215 µg/mL[6]	33.75%	Negative ± S9

[1] Study references are CCI
[2] The assay was performed twice; using triplicate plates
[3] Concentrations were selected based on preliminary cytotoxicity testing
[4] A 2.2-fold increase in the number of revertants was noted at 34 µg/ml with TA 100 in the first trial in the absence of S9, but was not considered biologically relevant in the absence of similar findings at higher doses and the lack of reproducible effects in the second trial. Sporadic increases in revertant colonies were noted for TA 98 and TA 1537 at low concentrations in the presence of S9. These were not considered biologically relevant as they were not dose-related nor reproducible.
[5] Duplicate cultures tested
[6] Maximum concentrations selected based on changes in osmolality

[3] https://www.uniprot.org/

Toxicity studies

13-week oral toxicity study in rats (CCI) Regulatory status: GLP; conducted in accordance with OECD Test Guideline (TG) 408 (1998)

In a 13-week oral toxicity study, thermolysin preparation was administered to Sprague Dawley rats by oral gavage at doses of 0, 84.45, 168.75 or 337.5 mg total organic solids (TOS)/kg bw/day. The test item was a liquid concentrate containing 23% sorbitol and 8% NaCl as a stabiliser and preservative, respectively, in water. Sorbitol and NaCl in water also served as the vehicle control. One mid dose female and two high dose females died during the study. Histopathological examination of these animals showed changes in the lungs indicating the deaths occurred due to oral gavage errors and were not related to treatment with the test item. There were no treatment-related clinical signs or adverse effects on any of the parameters evaluated. The no observed adverse effect level (NOAEL) in this study was 337.5 mg TOS/kg bw/day, the highest dose tested.

3.2.4 Potential for allergenicity

Searches for homology of the thermolysin amino acid sequence with those of known allergens were performed in 2023 by the applicant using the AllergenOnline database[4]. Searches included a search for 80 amino acid stretches within the sequence with > 35% identity to known allergens, which found no match to known allergens.

Based on the available information thermolysin is not expected to pose a risk of food allergenicity.

3.3 Safety assessments by other agencies

As noted in section 3.2.1 of this report, thermolysin from *A. caldiproteolyticus* Rokko is approved for use in multiple countries including France, Denmark, China and Mexico. Safety assessments from these jurisdictions are not available to FSANZ. The enzyme has also been self-affirmed as Generally Recognized as Safe (GRAS) in the USA, but the evaluation has not been reviewed by the US Food and Drug Administration (US FDA).

4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worst-case scenario' approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the thermolysin enzyme preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al. 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an acceptable daily intake (ADI) or a NOAEL to estimate a margin of exposure (MOE) for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020a). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

[4] http://www.allergenonline.org/

In their budget method calculation, the applicant made the following assumptions:

- all solid foods and non-milk beverages contain the highest use level of 163 mg TOS/kg in the raw material (proteins of various sources).
- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day.
- 50% of solid food is processed.
- the highest level of TOS in the final foods from all proposed uses in solid foods was used in the calculation (there was only one-use level proposed for non-milk beverages).
- among all solid food, protein bars produced the highest theoretical enzyme exposure when each solid food was assessed individually. Therefore the enzyme preparation use level and the raw material to final food ratio for this food was used in the budget method calculation to represent all solid foods.
- the maximum physiological requirement for liquid is 100 mL/kg body weight/day (the standard level used in a budget method calculation for non-milk beverages).
- 25% of non-milk beverages are processed.
- among all non-milk beverages, use in sports drink was the only use presented for beverages. Therefore the level of TOS from the enzyme preparation use for sports drink was used in the budget method calculation for all processed non-milk beverages.
- all of the TOS from the enzyme preparation remains in the final food.
- the final foods containing the theoretical amount of the thermolysin enzyme would be consumed daily over the course of a lifetime.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 1.833 mg TOS/kg body weight/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure:

- The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2020a). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst-case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations for solid food and non-milk beverages is 2.45 mg TOS/kg bw/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that all of the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that the enzyme is either not present in the final food, or present in insignificant quantities having no function or technical effect in the final food. In addition, the enzyme would be inactivated and perform no function in the final food to which the ingredient is added.

5 Discussion

The use of Thermolysin from *A. caldiproteolyticus* Rokko as a processing aid for protein hydrolysis is consistent with its functions and the typical functions of other proteases. It will be used in the manufacture and/or processing of dairy foods, eggs, meat and fish, protein concentrates and isolates, yeast and in beer brewing where it may confer functional benefits including flavour improvement, improved physical properties, increased yields (e.g. extracts), and processing efficiencies.

Thermolysin is functioning as a processing aid for the purposes of the Code and does not perform a technological purpose in the food for sale. The evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified.

No public health or safety risks were identified during the microbiological risk assessment.

Thermolysin from *A. caldiproteolyticus* Rokko has been used in some countries overseas for more than 10 years. No adverse effects have been reported. Bioinformatics analysis found no significant homology of the enzyme with known toxins or food allergens.

Thermolysin was not genotoxic *in vitro* and did not cause adverse effects in a 13-week oral toxicity study in rats. The NOAEL in this study was 337.5 mg TOS/kg bw/day, the highest dose tested. The TMDI was calculated by FSANZ to be 2.45 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 100.

6 Conclusions from the risk and technical assessment

Based on the available evidence there are no public health and safety concerns associated with the proposed uses of thermolysin from *A. caldiproteolyticus* Rokko. Its use as processing aid is technologically justified. In the absence of any identifiable hazard an acceptable daily intake (ADI) 'not specified' is appropriate.

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