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

18 December 2025
373-25

Supporting Document
Risk and technical assessment

Application A1328 Aminopeptidase from *Trichoderma reesei* as a processing aid

Executive summary

Food Standards Australia New Zealand (FSANZ) received an application from IFF Australia Pty Ltd (Trading as Danisco Australia Pty Ltd) to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of aminopeptidase Y (EC 3.4.11.15) from *Trichoderma reesei,* containing the gene for aminopeptidase Y from *Aspergillus clavatus,* as a processing aid in protein and yeast processing and flavour production.

The proposed use of this aminopeptidase Y as an enzyme processing aid in the quantity and form proposed is consistent with its typical function. Aminopeptidase Y performs its technological purpose during food processing but does not perform its technological purpose in food for sale. Therefore, it functions as a processing aid for the purposes of the Code. This aminopeptidase Y is not protein-engineered.

The enzyme preparation meets relevant identity and purity specifications.

The microbiological assessment undertaken by FSANZ did not identify any public health and safety concerns associated with using *T. reesei* as a source of aminopeptidase Y. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

The aminopeptidase Y sequence does not share homology with any known toxin, venom or allergen. No evidence of genotoxicity was found in a bacterial reverse mutation assay or an *in vitro* mammalian chromosomal aberration assay. No adverse effects were identified in a 90-day oral gavage study in rats, in which the no observed adverse effect level (NOAEL) was 1000 mg total organic solids (TOS)/kg bw/day, the highest dose level tested.

The theoretical maximum daily intake (TMDI) of aminopeptidase Y was calculated to be 9.03 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, an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate in the absence of any identifiable hazard.

FSANZ concludes that no public health and safety concerns are associated with using aminopeptidase Y derived from *T. reesei* in the quantity and form consistent with its typical function as a processing aid in protein and yeast processing and flavour production.

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1. Introduction

Danisco Australia Pty Ltd has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of aminopeptidase Y (EC 3.4.11.15) from *Trichoderma reesei* containing the gene for aminopeptidase Y from *Aspergillus clavatus* as a processing aid.

The enzyme preparation is intended for use in protein and yeast processing and flavour production at the minimum level required to achieve the desired effect, following Good Manufacturing Practice (GMP) principles.

The objectives of this comprehensive risk and technical assessment were to:

- Determine whether the proposed purpose is solely technological and whether the enzyme preparation effectively achieves its technological purpose as a processing aid in the proposed quantity and form.
- Evaluate potential public health and safety concerns associated with using this food enzyme preparation by considering the safety and history of use of the production organism, as well as the safety of the enzyme itself.

Some information evaluated by FSANZ is confidential commercial information and therefore is protected from disclosure under the *Food Standards Australia New Zealand Act 1991* (the Act)

2. Food technology assessment

2.1. Identity of the enzyme

Aminopeptidase Y (EC 3.4.11.15) is an enzyme in the peptidase family M28, specifically the class of metalloexopeptidases (Lothar and Matthews, 2002).

Aminopeptidase Y cleaves amino acids from the N-terminus of peptides and proteins (Figure 1). It targets neutral or hydrophobic amino acids, like leucine and phenylalanine (Sjöströmet *et al*., 2002).

2.1.1. Aminopeptidase Y (EC 3.4.11.15)

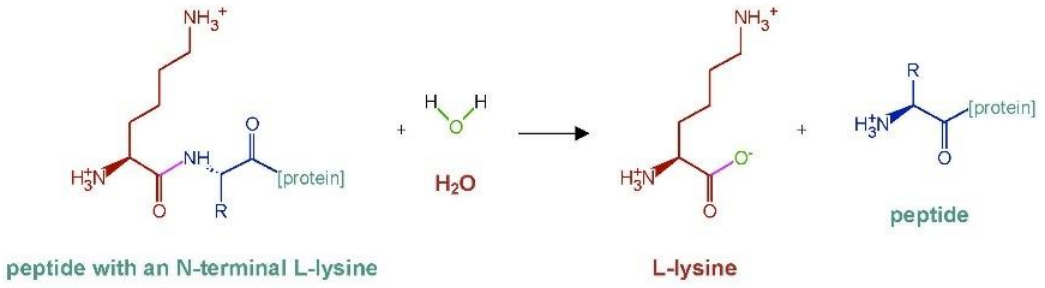
The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using the IUBMB[1] Enzyme Nomenclature Reference Database (McDonald *et al*., 2009). The identity of the enzyme was confirmed using ExplorEnz[2], the IUBMB Enzyme Nomenclature and Classification List. Details of the identity of the enzyme are provided in Table 1.

Table 1. Identity of Aminopeptidase Y (EC 3.4.11.15)

Accepted IUBMB name:	aminopeptidase Y
Other names/common names:	aminopeptidase Co; aminopeptidase (cobalt-activated); lysyl aminopeptidase
IUBMB enzyme nomenclature:	EC 3.4.11.15
ECTree	3. Hydrolases 3.4 Acting on peptide bonds (peptidases 3.4.11 Aminopeptidases 3.4.11.15 aminopeptidase Y
CAS number:	114796-97-3
Reaction:	Preferentially, release of N-terminal lysine (Figure 1)
Comments	Requires Co^{2+}; inhibited by Zn^{2+} and Mn^{2+}. An enzyme best known from *Saccharomyces cerevisiae* that hydrolyses Lys-$NHPhNO_2$ and, more slowly, Arg-$NHPhNO_2$. Type example of peptidase family M28

[1] International Union of Biochemistry and Molecular Biology.

[2] ExplorEnz: Official IUBMB Enzyme List (enzyme-database.org)



Source: MetaCyc database https://biocyc.org/reaction?orgid=META&id=RXN-18719

Figure 1. Preferential release of N-terminal lysine

2.2. Manufacturing process

2.2.1. Production of the enzyme

Aminopeptidase Y is produced by submerged fermentation of *T. reesei* carrying the aminopeptidase Y gene from *A. clavatus.*

The applicant's information demonstrated that their aminopeptidase is produced using a typical industrial process, following current Good Manufacturing Practice (cGMP) guidelines for Food and the principles of Hazard Analysis and Critical Control Point (HACCP). All raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards.

The application provided details on the manufacturing process, raw materials, and ingredients used in producing the applicant's aminopeptidase Y preparation, some of which are confidential commercial information (CCI). The aminopeptidase Y is not protein engineered.

2.2.2. Specifications for identity and purity

There are international general specifications for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO JECFA Monographs 26 2021; FAO/WHO 2006) and the Food Chemicals Codex (FCC, 2022). Enzymes used as processing aids must meet these specifications.

Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within the specifications in sections S3—2 or S3—3. The enzyme preparation does not exceed maximum levels for arsenic, cadmium, mercury (≤1 mg/kg) and lead (≤2 mg/kg) in sections S3—4.

The applicant provided analytical results of different batches of aminopeptidase Y. Table 2 compares the results of these analyses with international specifications established by JECFA and those outlined in the Code, where applicable.

Based on those results, the enzyme preparation met all relevant specifications.

Table 2. Comparison of the manufacturer's enzyme preparation with JECFA, Food Chemicals Codex, and Code enzyme specifications.

Parameters	Batch Analyses	Specifications	
		JECFA[1]	The Code[2]
Enzyme activity (units/g)	≥2600	-	-
Lead (mg/kg)	<0.5	≤5	≤2
Arsenic (mg/kg)	<0.5	-	≤1
Cadmium (mg/kg)	<0.1	-	≤1
Mercury (mg/kg)	<0.1	-	≤1
Total viable count (cfu[3]/mL)	41	-	-
Total coliforms (cfu/mL)	<1	≤30	-
Salmonella (in 25 mL)	Absent	Absent	-
Escherichia coli (in 25 mL)	Absent	Absent	-
Mycotoxin /mL	Negative	-	-
Antibiotic activity	Negative	Absent	

[1]Joint FAO/WHO Expert Committee on Food Additives (FAO JECFA Monographs 26 (2021), [2]The code, Section S3—4, [4]cfu = colony forming units

2.3. Technological purpose

Enzyme preparations, including peptides such as aminopeptidase Y, are widely used as processing aids in manufacturing food products. They assist in processing but have no function in the final product. Heat often denatures or inactivates them during further processing (Fisher, 2004; Kumar *et al*., 2024). Aminopeptidase Y has potential processing aid applications in the food industry, especially in areas involving protein hydrolysis, flavour enhancement, and fermentation (EFSA, 2024).

The applicant's aminopeptidase Y aids protein and yeast processing and flavour production, and they requested that the enzyme preparation be used at GMP levels. Aminopeptidase Y releases free amino acids from peptides, particularly hydrophobic ones such as leucine and phenylalanine, which serve as precursors to aroma compounds. This enzyme creates the "umami" taste and enhances overall palate richness in aged foods (Nandan and Nampoothiri, 2020). In the baking industry, proteases are added to flour at the mill or the bakery to aid in protein and yeast processing. Additionally, proteases found in the yeast used for leavening bread play an important role in yeast processing (Philipps-Wiemann, 2018).

When used in creating protein hydrolysates, aminopeptidase Y helps achieve more complete hydrolysis, resulting in peptides and free amino acids that may be more digestible (Wang *et al.,* 2023)

In beer brewing or wine fermentation, aminopeptidases aid protein and yeast processing and flavour production. The enzyme can assist in breaking down peptides, making nitrogen sources more accessible to yeast and improving fermentation performance (Spier *et al*., 2016; Souza *et al*., 2023).

The applicant provided sufficient data on their enzyme preparation's physical and chemical

properties to demonstrate thermostability and pH stability when used for the intended technological purpose; this data was CCI.

2.4. Food Technology Conclusion

- The use of this aminopeptidase Y as a processing aid in protein, yeast processing, and flavour production is consistent with its typical function as a hydrolase acting on peptide bonds.
- The aminopeptidase serves its technological purpose as a hydrolase, after which it does not perform a technological function in the final food product. It therefore functions as a processing aid for the purposes of the Code.
- The Code includes relevant identity and purity specifications for the enzyme, and the applicant has provided evidence that the enzyme preparation meets these specifications.

3. Safety Assessment

This safety assessment aims to evaluate potential public health and safety concerns arising from using this aminopeptidase Y associated with *T. reesei* as a processing aid.

Some information relevant to this section is CCI, so full details cannot be disclosed under the Act.

3.1. Source microorganism

FSANZ has previously assessed the safety of *T. reesei* as the source organism for at least 15 processing aids in Schedule 18. Several enzymes produced by *T. reesei* QM6a have Generally Recognized as Safe (GRAS) status with the Food and Drug Administration (FDA) or FDA had no questions about the GRAS conclusions about them contained in GRAS submissions to FDA (USEPA 2012).

Trichoderma reesei is a biosafety level 1, common, hypercellulolytic, soil fungus that was initially isolated from deteriorating canvas made from cellulosic material. The production organism used by the applicant is a derivative of *T.* reesei strain QM6a. Strain QM6a is the wild type of the majority of *T. reesei* industrial production strains (Nevalainen et al. 1994). Strain QM6a is the type strain for *T. reesei* and has been registered with the American Type Culture Collection under ATCC13631 (Olempska-Beer et al. 2006).

T. reesei has a history of safe use in industrial-scale enzyme production (Nevalainen et al. 1994, Blumenthal 2004, Nevalainen and Peterson 2014, Paloheimo et al. 2016, Frisvad et al. 2018). Food enzymes derived from *T. reesei* strains (including recombinant *T. reesei* strains) have been evaluated by JECFA and many countries which regulate the use of food enzymes, such as Australia, the USA, France, Denmark and Canada.

T. reesei QM6a strains are non-pathogenic, not known to possess any virulence factors associated with colonisation or disease, and do not present any human toxicity concerns (USEPA 2012). Although some Trichoderma species can produce various mycotoxins and antifungal metabolites, several review papers support the safety of *T. reesei* QM6a strains with no production of known mycotoxins or antibiotics under conditions used for enzyme production (Nevalainen et al. 1994, Kubicek et al. 2007, Peterson and Nevalainen 2012, Frisvad et al. 2018). *T. reesei* QM6a strains are known to produce the peptaibol antibiotic paracelsin, but industry-standard submerged fermentation conditions are not linked to the production of paracelsin (USEPA 2012).

The applicant provided data that adequately demonstrates the production strain's identity as a derivative of *T. reesei* QM6a. Microbiological testing was provided to FSANZ confirming the absence of the production organism and toxicologically significant amounts of mycotoxins in the final enzyme preparation.

The microbiological assessment undertaken by FSANZ did not identify any public health and safety concerns related to *T. reesei* QM6a or its derivatives as a source organism for aminopeptidase Y.

3.2. Gene donor organism

The gene for aminopeptidase Y was synthetically constructed based on the published amino acid sequence from *A. clavatus*. As this process did not involve the direct use of genetic material from *A. clavatus*, there is no possibility of any extraneous DNA from the donor organism being present in the production strain.

3.3. Description of the DNA to be introduced and the method of transformation

The published amino acid sequence of aminopeptidase Y from *A. clavatus* was used to synthesize the genetic material to be expressed by *T. reesei.* The inserted gene was placed under the control of a promoter and terminator from *T. reesei*. Copies of the expression cassette were integrated into the *T. reesei* chromosome using standard molecular biology techniques.

3.4. Characterisation of the inserted DNA

Next generation sequencing of the production strain confirmed the insertion of the expression cassette in the genome at a complex integration site.

3.5. Genetic stability of the inserted gene

The genetic stability of the production strain was confirmed by genome sequencing. Samples of broth were collected before and after prolonged fermentation that was designed to mimic commercial fermentation conditions. The samples were used for genomic DNA extraction and next generation sequencing. No changes were observed between pre- and post-fermentation samples, demonstrating that the insertion cassette had been stably maintained over multiple generations.

3.6. Safety of the enzyme

3.6.1. History of safe use

The applicant provided documentation demonstrating that the enzyme has been approved for use in four countries, including the USA, to which the USFDA responded with a "No Questions" letter to a GRAS notification.

FSANZ notes that aminopeptidase from *Aspergillus oryzae* is approved in the Code and is approximately 71% homologous to aminopeptidase Y from *Aspergillus clavatus*.

3.6.2. Bioinformatic assessment of homology with known toxins

A BLAST search for homology of the aminopeptidase Y sequence against the complete Uniprot[3] Database was performed, with a threshold E-value of 0.1. No matches annotated as either a toxin or a venom were found. Most matches were peptide hydrolases. An additional, specific BLAST search for homology of the aminopeptidase Y sequence was performed against the Uniprot animal toxin database. This yielded no matches.

3.6.3. Toxicology data

3.6.3.1. *Genotoxicity studies*

The applicant provided study reports of two genotoxicity studies with the aminopeptidase Y preparation. Both studies were conducted under GLP and according to relevant OECD Test Guidelines. Appropriate positive controls in these studies produced the expected responses, confirming the validity of the assays. The results of these studies, as summarised in Table 3,

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

showed no evidence of mutagenicity, clastogenicity or aneugenicity.

Table 3. ***Genotoxicity studies of aminopeptidase from* Trichoderma reesei**

Test	*Test system*	*Concentration*	*Purity (% total organic solids)*	*Results*
Bacterial reverse mutation assay (OECD TG 471, [1997])	*Salmonella enteridis* var. Typhimurium test strains TA98, TA100, TA1535 and TA1537; and *Escherichia coli* strain WP2 *uvr*A	Experiment I[1]: 1.50, 5.00, 15.0, 50.0, 150, 500, 1500 and 5000 µg per plate Experiment II[2]: 5.0, 50.0, 150, 500, 1500 and 5000 µg per plate	27.84%	Negative ± S9
***In Vitro* Mammalian Chromosomal Aberration Assay in Human Peripheral Blood Lymphocytes (OECD TG 473 [2016])**	Cultured human peripheral blood lymphocytes[3]	4+16 hour -S9: 625, 250, 2500, 5000 µg/mL 4+16 hour +S9: 625, 250, 2500, 5000 µg/mL 20 hour -S9: 625, 1250, 2500, 5000 µg/mL	27.84% w/w	Negative ± S9

[1] Test conducted in duplicate.
[2] Test conducted in triplicate.
[3] Lymphocytes obtained from one healthy female donor.

3.6.3.2. *Toxicity studies*

90-day oral gavage study of aminopeptidase Y in Sprague-Dawley rats. Regulatory status: GLP, compliant with OECD Test Guideline 408 (2018)

The aminopeptidase Y used in this study comprised 27.84% total organic solids (TOS) and was administered to the rats (10/sex/group) at doses of 0, 250, 500 or 1000 mg TOS/kg bw/day.

Rats were weighed weekly, and feed consumption was recorded weekly. Rats were subject to twice-daily cage-side checks and weekly detailed examinations. Ophthalmic examinations and neurobehavioural evaluations were conducted pre-study and during the last week of the in-life phase. Blood and urine were collected at the end of the in-life phase, after which rats were terminated for detailed necropsy. Weights of selected organs were recorded, and tissues and organs were preserved for microscopic examination.

All rats survived to the end of the in-life phase. There were no treatment-related effects on clinical signs, ophthalmic findings, or group mean values for bodyweight, bodyweight gain, feed consumption, neurobehavioural findings, haematological parameters, clinical chemistry parameters, urinalysis parameters, or organ weights. There were no treatment-related gross or microscopic lesions found. The =no observed adverse effect level was 1000 mg TOS/kg bw/day, the highest dose tested.

3.6.4. Potential for allergenicity

The applicant provided results of sequence homology searches conducted of the Food Allergy Research and Resource Program (FARRP) AllergenOnline database Version 22 (May 2023). The searches included a full-length sequence alignment, a sliding window of 80 amino acid sequences, and a search for an exact 8 amino acid match. No matches to known allergens were identified in any of the searches.

The applicant also provided an allergen declaration, which indicates that the glucose used in the fermentation process is derived from wheat.

3.6.5. Assessments by other regulatory agencies

No assessments by other national or international regulatory agencies are available for this aminopeptidase Y.

3.7. 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 aminopeptidase 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 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 2020). The method is used by international regulatory bodies and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

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

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed
- among all proposed uses in different solid foods, protein hydrolysates 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 protein hydrolysates was used in the budget method calculation to represent all solid foods.
- all solid foods contain the highest use level of 180.6 mg TOS/kg in the final food
- 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)
- there is no exposure to TOS from the enzyme preparation from non-milk beverages
- all of the TOS from the enzyme preparation remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 2.26 mg TOS/kg bw/day in the final food. FSANZ notes this calculation does not include the TOS from non-milk beverages.

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 2009). 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.
- If approved for the stated purposes, the enzyme could potentially be used in production of non-milk beverages. As such, FSANZ has taken the conservative approach of including exposure from non-milk beverages in its calculations at the same use level that was used for solid foods.

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 is 9.03 mg TOS/kg bw/day.

Both FSANZ and the 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 despite the applicant stating that it is likely to either be absent in the final food or the inactive residue would be present in negligible amounts.

3.8. Safety Assessment Conclusions

No public health or safety concerns were identified concerning the production organism. Analysis of the production strain confirmed the presence and stability of the inserted DNA.

The aminopeptidase sequence does not share homology with any known toxin or venom, or with any known allergen. No evidence of genotoxicity was found in a bacterial reverse mutation assay or in an *in vitro* mammalian chromosomal aberration assay. No adverse effects were identified in a 90-day oral gavage study in rats, in which the NOAEL was 1000 mg TOS/kg bw/day, the highest dose level tested. The TMDI was calculated by FSANZ to be 9.03 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a MOE of approximately 100.

Based on the reviewed data, an Acceptable Daily Intake (ADI) 'not specified' is appropriate in the absence of any identifiable hazard.

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