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**197-22**

## **Supporting document 1**

Risk and technical assessment – Application A1238

Serine endopeptidase enzyme from GM *Trichoderma reesei*

### **Executive summary**

AB Enzymes applied to Food Standards Australia New Zealand (FSANZ) to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include thermomycolin (EC 3.4.21.65), a protease of the serine endopeptidases class, as a processing aid. It is produced from a genetically modified (GM) strain of *Trichoderma reesei* containing the thermomycolin gene from *Malbranchea cinnamomea*. The specific name for the production organism is *T. reesei* RF8963. The proposed use of thermomycolin is as a processing aid in the manufacture of meat, vegetable and seafood products.

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological purpose in the quantity and form proposed to be used and to evaluate public health and safety concerns that may arise from the use of this enzyme.

FSANZ concludes that the proposed use of thermomycolin as an enzyme in the production of meat, vegetable and seafood products is consistent with its typical function of hydrolysing proteins in those foods. Analysis of the evidence provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used and consistent with Good Manufacturing Practice (GMP) controls and processes, is technologically justified.

Thermomycolin performs its technological purpose during the production of proteinaceous foods and is not performing a technological purpose in the final food, therefore functioning as a processing aid as defined in the Code. There are relevant identity and purity specifications for the enzyme in the Code.

No public health and safety concerns were identified in the assessment of thermomycolin from a modified strain of *T. reesei* under the proposed use conditions.

The host is neither pathogenic nor toxigenic and analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro*. The no observed adverse effect level (NOAEL) in a 90-day oral gavage study in rats was 1000 mg/kg bw/day enzyme, which was the highest dose tested and is equivalent to 150 mg/kg bw/day total organic solids (TOS). The theoretical maximum daily intake (TMDI) was calculated by FSANZ to be 0.085 mg/kg bw/day TOS. Comparison of the NOAEL and the TMDI gives a margin of exposure (MOE) of approximately 1760.

Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins or venoms. However a degree of homology between the enzyme and several

allergens was found. Taking into account that none of these allergens are food allergens, the enzyme was shown to be susceptible to proteolysis by digestive enzymes and only low levels of the enzyme processing aid are expected to be present in final food products, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

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

The applicant, AB Enzymes, is seeking permission for thermomycolin (EC 3.4.21.65), a protease of the serine endopeptidases class, as a processing aid. The enzyme is produced from a genetically modified (GM) strain of *Trichoderma reesei* containing the thermomycolin gene from *Malbranchea cinnamomea*. The specific name for the production organism is *T. reesei* RF8963.

AB Enzymes' thermomycolin is a thermotolerant protease, suitable for catalysing the hydrolysis of peptide bonds under mildly alkaline conditions. It is used in the manufacture of vegetable and animal protein hydrolysates including meat, poultry and game products, vegetable products, fish and seafood products.

Marketed as a liquid enzyme preparation, if approved, thermomycolin will be used as a processing aid at low levels and is either not present in the final food or present in insignificant quantities, having no technical function in the final food.

## 1.1 Objectives of the assessment

The objectives of this Risk and Technical assessment were to:

- determine whether the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used, and
- evaluate potential public health and safety issues that may arise from the use of this enzyme, produced by a genetically modified organism, as a processing aid, specifically by considering the:
  - history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s)
  - safety of the enzyme.

## 2 Food technology assessment

### 2.1 Characterisation of the enzyme

#### 2.1.1 Identity of the enzyme

The use of proteases for hydrolysing proteins during the manufacture of fish, seafood, animal and vegetable products has been extensive for many years in a number of EU countries and globally. AB Enzymes provided relevant information regarding the identity of the specific serine endopeptidase enzyme, thermomycolin. FSANZ verified this using an appropriate enzyme nomenclature reference (IUBMB 2022). Details of the identity of the enzyme are provided in Table 1.

**Table 1** Identity

<b>Generic common name:</b>	Thermomycolin
<b>Accepted IUBMB<sup>1</sup> name:</b>	Thermomycolin
<b>Systematic name:</b>	Serine endopeptidase/serine protease
<b>Other names:</b>	Thermomycolase, Proteinase, <i>Malbranchea pulchella</i> sulfurea extracellular
<b>EC number:</b>	3.4.21.65 (formerly covered by 3.4.21.14)
<b>CAS<sup>2</sup> registry number:</b>	52233-31-5
<b>Reaction:</b>	Nonspecific hydrolysis of proteins. Exhibits preferential cleavage at Alanine, Tyrosine and Phenylalanine in small molecule substrates

Thermomycolin is a serine endopeptidase (IUBMB 2022), which are commonly referred to as proteases (Campbell-Platt, 2018; Nagodawithana and Reed, 1993). Thermomycolin was originally part of a class of enzymes numbered by the IUBMB as EC 3.4.21.14. The IUBMB has since transferred this to individual entries, including for thermomycolin (EC 3.4.21.65), instead of the original EC number, EC 3.4.21.14 (IUBMB 2022).

Thermomycolin from *T. reesei* containing the thermomycolin gene from *M. cinnamomea* as requested in this application, is not currently permitted for use as a processing aid in the Code.

### 2.2 Manufacturing process

#### 2.2.1 Production of the enzyme

AB Enzymes' thermomycolin is produced by submerged fermentation which is a common method for producing food enzymes. The main fermentation steps are: inoculum; seed fermentation; main fermentation followed by the recovery stage involving primary and liquid separation; concentration to achieve the desired enzyme activity followed by polish and germ

<sup>1</sup> International Union of Biochemistry and Molecular Biology

<sup>2</sup> Chemical Abstracts Service

filtration. This provides a concentrated enzyme solution free of the production strain and insoluble substances. The final formulation of the enzyme preparation and associated packing follows. A manufacturing flow-chart was provided as an appendix with the application. Manufacturing occurs in accordance with current Good Manufacturing Practices for Food and the principals of Hazard Analysis of Critical Control Points. Compliance with Food Hygiene Regulation by relevant food inspection services in Finland also provides assurance.

The raw materials used in the fermentation and recovery processes are of food grade quality that meet predefined quality standards controlled by Quality Assurance for ROAL Oy<sup>3</sup>. The raw materials conform to either specifications set out in the Food Chemical Codex, 12th edition (2020) or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food. FSANZ notes that wheat products are utilised in the production of this enzyme.

The enzyme preparation is a liquid, consisting of glycerol, sorbitol, sodium benzoate and water and marketed under the trade name COROLASE® 8000. Whilst the manufacturing processes ensure the production microorganism is removed from the final enzyme preparation, the food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. The final enzyme preparation is produced to ensure it meets international purity specifications of enzymes, being the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the Food Chemicals Codex (FCC) (USP, 2020) as discussed in the next section.

### **2.2.2 Specifications**

There are international specifications for enzyme preparations used in the production of food. These have been established by JECFA in its Compendium of Food Additive Specifications and in the FCC. These specifications are included in the primary sources listed in section S3—2 of Schedule 3 of the Code. Enzymes used as a processing aid must meet either of 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 specifications in sections S3—2 or S3—3.

AB Enzymes provided the results of analysis of three different batches of the liquid enzyme preparation. Table 2 provides a comparison of these batches with the international specifications established by JECFA and FCC, as well as those detailed in the Code (being section S3—4, as applicable).

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<sup>3</sup> A major manufacturer of enzymes for industrial applications such as baking, food, technical and feed industries.

**Table 2** Comparison of AB Enzymes' Thermomycolin liquid enzyme preparation compared to JECFA, Food Chemicals Codex and Code specifications for enzymes

Analysis	Analysis provided by manufacturer*	Specifications		
		JECFA (2006)	Food Chemicals Codex (FCC, 2020)	Australia New Zealand Food Standards Code (section S3—4)
Lead (mg/kg)	<0.05	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.5	-	-	≤1
Cadmium (mg/kg)	<0.5	-	-	≤1
Mercury (mg/kg)	<0.5	-	-	≤1
Coliforms (cfu/g)	<30	≤30	≤30	-
Salmonella (in 25 g)	Not detected	Absent	Negative	-
E. coli (in 25 g)	Not detected	Absent	-	-
Antibiotic activity	Not detected	Absent	-	-

Based on the above results, the enzyme preparation meets international and Code specifications for enzymes used in food production.

### 2.3 Technological purpose of the enzyme

Thermomycolin facilitates partial or extensive hydrolysis in protein processing, mainly in fish and meat, including casein, whey, gluten, and proteins from meat, fish, corn, soy, rice, peas, lentils etc. The resulting peptides are used as ingredients in a variety of food products, providing functional properties such as solubility, emulsification, gelling and foaming or increasing the protein content in foods for nutritional purposes.

Thermomycolin performs its technological function during food processing, optimising the hydrolysis process. Due to its thermostability, thermomycolin exhibits activity up to temperatures of 80°C. This can also reduce the risk of microbial contamination during processing. Denatured by high temperature at the end of processing, the enzyme provides no residual enzymatic activity in the final food. The physical and chemical properties of thermomycolin are presented in Table 3.

**Table 3** Thermomycolin enzyme preparation physical/chemical properties

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	100,000 BPU/g (Ave 123,000 over 3 batch results provided)
Appearance	Brown coloured liquid
Specific gravity	1.15-1.30
Temperature stability	Optimum at up to 80°C.
pH range	Max activity within range 6.0 – 10.0
Storage stability	24 months at less than 10°C

\* BPU: Assay of protease activity with TCA, Third party internal method.

## 2.4 Technological justification

Thermomycolin provides production consistency and economies, increased yield, enhanced flavours. Thermomycolin can be utilised on a wide range of substrates, specifically vegetable derived raw materials such as soy, wheat, maize etc., animal derived raw materials such as milk and milk derived products (whey proteins, caseins), meat, fish, collagen and gelatine.

The applicant states that during fish processing, thermomycolin aids the primary hydrolysis by separating the protein fraction (fish hydrolysates) from the bones and oil fraction.

Thermomycolin benefits the production of concentrated fish hydrolysates by:

- improving liquid/solid separation process, leading to higher yield of soluble proteins and peptides
- improving the quality of the finished products and maximising the value of the trimmings
- allowing mild process conditions compared to alternative protein hydrolysis processes such as alkaline, acid hydrolysis or heat treatment
- reducing viscosity, leading to improvement of the overall processing of fish products, improving the evaporation rate of the fish hydrolysate, providing a higher solids proportion, reduced energy consumption during drying and reduced scaling of equipment, hence reduced cleaning cycles.

During meat processing, thermomycolin is used to facilitate protein hydrolysis in meat by-products (e.g. bone cleaning, gelatine production etc.) and the production of meat hydrolysates (meat or bone stocks, hydrolysed animal proteins).

The benefits of thermomycolin in meat processing are similar to those described above for fish processing. In addition, for gelatine production, the applicant states the benefits as:

- providing a significant cost reduction and capacity increase through reduction in soaking and liming times
- increasing the gelatine yield
- improving the raw material quality by way of cleaner bones.

The stated technological purpose of serine endopeptidases, including thermomycolin, is supported by scientific literature (Damodaran et al, 2008; Nagodawithana and Reed, 1993).

Thermomycolin performs its technological function during the hydrolysis of the various substrates only. It is inactivated during the evaporation/concentration stage of the protein hydrolysate production process, where temperatures in exceed 85°C and by the subsequent pasteurisation or sterilisation steps.

## 2.5 Food technology conclusion

The proposed use of the enzyme is in the manufacture of fish, seafood, animal and vegetable products. FSANZ concludes that the evidence presented to support the proposed use provides adequate assurance that the enzyme, in the form and prescribed amounts, which must be consistent with GMP controls and processes, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

The enzyme performs its technological purpose during production and manufacture of foods after which it is inactivated, thereby not performing a technological function in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code.



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

### **3 Safety assessment**

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by this microorganism, as a processing aid.

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

#### **3.1 History of use**

##### **3.1.1 Host organism**

FSANZ has previously assessed the safety of *T. reesei* as the host organism for a number of enzymes used as processing aids. Schedule 18 to Standard 1.3.3 of the Code permits the use of the following enzymes derived from genetically modified *T. reesei* strains including: Endo-1,4- $\beta$ -xylanase, Lysophospholipase, Aspergillopepsin I,  $\alpha$ -Glucosidase, Glucose oxidase, Glucoamylase and  $\alpha$ -Amylase. The taxonomy was confirmed by a recognised culture collection.

##### **3.1.2 Gene donor organisms**

The gene sequence for the thermomycolin enzyme was sourced from *M. cinnamomea*. This organism is a filamentous fungus found in soil and rotting plant material. Synonyms include *M. pulchella* var. *sulfurea* and *M. sulfurea*. Identification of the organism was determined using standard morphological and molecular methods.

#### **3.2 Characterisation of the genetic modification(s)**

##### **3.2.1 Description of the DNA to be introduced and method of transformation**

The thermomycolin gene from *M. cinnamomea* was used to construct an expression cassette in the pUC19 plasmid vector using standard molecular biology techniques. The constructed expression cassette contained the thermomycolin, under the control of a *T. reesei* promoter and terminator, and the *Aspergillus nidulans amdS* selectable marker gene under the control of the *A. nidulans amdS* promoter and terminator. The *amdS* marker allowed for selection of positive transformants by growth on media containing acetamide (Kelly and Hynes 1985).

The expression cassette was removed from the construction plasmid using restriction enzyme digestion and agarose gel electrophoresis, prior to transformation into the *T. reesei* host strain using PEG-mediated protoplast fusion as described by Penttilä et al. (1987) with the modifications described in Karhunen et al. (1993).

##### **3.2.2 Characterisation of inserted DNA**

Southern blot analysis using a probe targeting the *M. cinnamomea* thermomycolin gene sequence was performed on the genome of the *T. reesei* production strain. Results indicated that several copies of the expression cassettes were integrated in the genome of *T. reesei* RF8963

### 3.2.3 Genetic stability of the inserted gene

The stability of the introduced DNA in the production strain was demonstrated by Southern blot analysis of genomic DNA samples extracted after three separate fermentation processes. These data indicated that the introduced thermomycolin gene is genetically stable in the *T. reesei* RF8963 genome and is suitable for industrial fermentation.

## 3.3 Safety of thermomycolin

### 3.3.1 History of safe use

Thermomycolin from *M. cinnamomea* produced in *T. reesei* is not an approved enzyme processing aid in the Code and does not have a history of safe use in Australia or New Zealand. Other serine proteinases from *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, *Bacillus halodurans*, *Bacillus licheniformis* and *Bacillus subtilis* are currently permitted in Schedule 18 of the Code. Thermomycolin from *M. cinnamomea* produced in *T. reesei* is approved for use in Denmark, France, Canada, USA, Brazil and Mexico.

There are no known reports of adverse effects arising from the use of thermomycolin or other serine proteinases in Australia or New Zealand, or any other jurisdiction where these enzymes have been approved as processing aids in food.

### 3.3.2 Bioinformatics concerning potential for toxicity

A BLAST search was performed (May 2019) using the mature amino acid sequence against the non-redundant sequences in the NCBI sequence database<sup>4</sup>. Based on the results obtained from these searches, it can be concluded that the thermomycolin from *M. cinnamomea* does not show significant homology to any protein sequence that would indicate toxicity.

An additional BLAST search was performed by FSANZ during risk assessment (January, 2022), using the translated amino acid sequence against the complete UniProt database<sup>5</sup>. No sequence matches in the top 1000 results were annotated as either toxins or venoms.

### 3.3.3 Evaluation of toxicity studies

The test item used in the following toxicity studies was *M. cinnamomea* thermomycolin produced using modified *T. reesei* RF8963 and is representative of the commercial enzyme product.

#### **Animal Studies**

90-day repeated dose oral toxicity study in rats (Eurofins BioPharma & BSL BIOSERVICE, 2017). Regulatory Status: GLP; conducted according to OECD Test Guideline (TG) 408 (1998).

The thermomycolin test item was administered to Wistar Crl:WI(Han) rats (10/sex/group) at doses of 0, 100, 300, 600 and 1000 mg/kg bw/day enzyme (0, 15, 45, 90 and 150 mg/kg bw/day total organic solids [TOS], respectively) by oral gavage for 13 weeks. The vehicle control was water.

Animals were observed daily for signs of toxicity. Body weight, food consumption and

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<sup>4</sup> NCBI sequence database: <https://blast.ncbi.nlm.nih.gov/>

<sup>5</sup> UniProt database: <https://www.uniprot.org/blast/>

detailed clinical examinations for signs of toxicity were recorded weekly. Ophthalmological examination and functional performance tests were conducted prior to first treatment and in week 13. Haematology, blood coagulation, clinical biochemistry, urinalysis, gross pathology, measurement of organ weights and histopathological examination was conducted on all animals at study termination.

No treatment related mortality or clinical signs were observed, nor treatment related differences in feed consumption, body weights, haematology, clinical chemistry, ophthalmology, or functional observations or motor activity parameters. No remarkable macroscopic or histopathological changes were observed at necropsy.

The no observed adverse effect level (NOAEL) was 1000 mg/kg bw/day enzyme (150 mg/kg bw/day TOS), the highest dose tested.

### **Genotoxicity**

*In vitro mammalian cell gene mutation test using the thymidine kinase gene (Eurofins BioPharma, 2016). Regulatory Status: GLP; conducted according to OECD TG 490.*

The potential mutagenicity of thermomycolin was evaluated in mouse lymphoma L5178Y (clone TK<sup>+/-</sup>-3.7.2C) cells, with and without metabolic activation using rat liver homogenate (S9). Cells were exposed to the thermomycolin test item for 4 hours at 8 different concentrations within the dose range of 250-2000 µg/mL in the presence of S9, or 50-1800 µg/mL in the absence of S9, determined using a preliminary dose selection experiment. Positive controls were tested in parallel using ethylmethanesulphonate and methylmethanesulfonate in the absence of S9, and benzo[a]pyrene in the presence of S9. Sterile culture medium was used as the vehicle control.

No biologically relevant increases in cell colonies were observed in mouse lymphoma L5178Y TK<sup>+/-</sup> cell cultures treated with the thermomycolin test item, with or without metabolic activation. All control treatments showed the anticipated mutagenic activity and were consistent with historical control ranges of the test facility, demonstrating the validity of the assay. It was concluded that the thermomycolin test item was not mutagenic under the conditions of the study.

*In vitro mammalian cell micronucleus test (Eurofins BioPharma, 2016). Regulatory status: GLP; conducted according to OECD TG 487.*

The potential of thermomycolin to increase micronuclei formation in mammalian cells was tested using human lymphocytes isolated from peripheral blood, collected from a healthy volunteer. Treatment with the thermomycolin test item was either a 4 hour pulse exposure with or without S9, followed by a 40 hour recovery; or 43-44 hours of continuous exposure without S9. Positive control assays were conducted in parallel using ethylmethanesulphonate and colchicine in the absence of S9 as clastogenic and aneugenic controls respectively, and cyclophosphamide as a clastogenic control in the presence of S9. Sterile culture medium was used as the vehicle control. The experiment was carried out once in duplicate.

Based on preliminary dose selection experiments, the dose range of 1000-2750 µg/mL and 2000-3500 µg/mL was used for the 4 hour pulse exposure treatment, with and without S9 respectively, and 25-400 µg/mL for the continuous exposure without S9.

There were no treatment related increases in micronuclei formation observed in peripheral blood lymphocytes following exposure to the thermomycolin test item, relative to the vehicle controls, under any of the conditions tested. The positive controls demonstrated a statistically significant increase in the formation of micronuclei, validating the sensitivity of the

experimental methodology. It was concluded that thermomycolin was not clastogenic or aneugenic under the conditions of the study.

### **3.3.5 Potential for allergenicity**

A FASTA search for homology to known allergens was performed using the amino acid sequence of thermomycolin using the AllergenOnline<sup>6</sup> database (queried January 2017) using two sequence alignments: an 80 mer sliding window (>35% homology) and the full length protein (>35% homology). FSANZ reaffirmed the bioinformatics searches in January 2022 utilising the sequence provided by the applicant.

None of the identified matches were recognised food allergens<sup>7</sup>. Proteinases from papaya, muskmelon (rock melon) and kiwi fruit are known food allergens, however these share low sequence similarity (less than 30% identity) with thermomycolin from *M. cinnamomea*. Furthermore, the applicant supplied the results of an *in vitro* digestion assay, showing that the thermomycolin was liable to pepsin proteolysis. Enzymes readily hydrolysed by proteolysis are generally considered to have a lower potential of becoming food allergens (FAO/WHO 2009a).

It was concluded that the presence of thermomycolin produced using modified *T. reesei* in food is unlikely to pose an allergenicity concern to consumers.

### **3.3.6 Assessments by other regulatory agencies**

Letters of approval for the use of *M. cinnamomea* thermomycolin produced using *T. reesei* in Denmark and France were provided by the applicant. These approvals were not accompanied by written assessments. The enzyme is also approved for use in Canada, USA, Brazil and Mexico.

The US FDA has responded with a 'no questions' letter to a self-assessment that thermomycolin produced using *T. reesei* is generally recognised as safe (GRAS) in the USA (GRN 817). A GRAS notification is not accepted by FSANZ as an assessment by the FDA.

## **3.4 Dietary exposure assessment**

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worse-case scenario' approach to estimating likely levels of dietary exposure assuming all added thermomycolin enzyme from GM *Trichoderma reesei* remained in the food.

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<sup>6</sup> AllergenOnline: <http://www.allergenonline.org/>

<sup>7</sup> World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee: <http://www.allergen.org/>

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 for risk characterisation purposes.

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

- the maximum physiological requirement of solid foods (including milk) is 25 g/kg body weight/day
- the maximum physiological requirement for non-milk beverages is 100 mL/kg body weight/day (the standard level used in a budget method calculation)
- 50% of solid food and 25% of non-milk beverages contain thermomycolin
- the maximum thermomycolin level in both final solid food and non-milk beverage was 1.7 mg TOS/kg food (i.e. the highest use level from all uses within each group)
- all of the enzyme remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of thermomycolin to be 0.064 mg TOS/kg body weight/day.

As assumptions made by the applicant differ to those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following different 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. This is the standard level used in a budget method calculation where there is potential for the enzyme to be in baby foods or general purpose foods that would be consumed by infants (FAO/WHO, 2009b), which for this enzyme would be from the products containing gelatine (dairy products, confectionary and gelatine desserts) and other animal protein hydrolysates.
- 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 2009b). 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 as a worst case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI based on FSANZ's calculations for both solid food and non-milk beverages was 0.0425 mg TOS/kg body weight/day each resulting in a total of 0.085mg TOS/kg bw/day.

Both the FSANZ and applicants estimates of the TMDI will be overestimates of the dietary exposure given the conservatism of the budget methods. This includes that it was assumed that the enzyme remains in the final foods and beverages. The applicant has stated that it is inactivated by heat in a specific inactivation step, in a sterilisation/pasteurization process or during further drying steps and does not have a function in the final food.

## 4 Discussion

No public health and safety concerns were identified in the assessment of thermomycolin from a modified strain of *T. reesei* under the proposed use conditions.

The host is neither pathogenic nor toxigenic and analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro*. The NOAEL in a 90-day oral gavage study in rats was 1000 mg/kg bw/day enzyme, which was the highest dose tested and is equivalent to 150 mg/kg bw/day TOS. The TMDI was calculated by the applicant to be 0.064 mg/kg bw/day TOS and independently calculated by FSANZ to be 0.085 mg/kg bw/day TOS. Comparison of the NOAEL and the TMDIs calculated by the applicant and FSANZ gives a margin of exposure (MOE) of approximately 2350 and 1760, respectively.

Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins or venoms. However a degree of homology between the enzyme and several allergens was found. Taking into account that none of these allergens are food allergens, the enzyme was shown to be susceptible to proteolysis by digestive enzymes and that only low levels of the enzyme processing aid are expected to be present in final food products, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

## 5 Conclusion

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an ADI 'not specified' is appropriate.

## 6 References

Campbell-Platt, G (2018) Food Science and Technology (second edition), John Wiley and Sons, West Sussex

Damodaran S, Parkin KL, Fennema OR (2008) Fennema's Food Chemistry (4th ed). Boca Raton: CRC Press/Taylor & Francis

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