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

12 December 2024
321–24

Supporting document

Risk and technical assessment – Application 1311

Prolyl oligopeptidase from GM *Trichoderma reesei* as a processing aid

---

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 prolyl oligopeptidase, EC 3.4.21.26. This prolyl oligopeptidase is produced from a genetically modified (GM) strain of *Trichoderma reesei* containing the prolyl oligopeptidase gene from *Aspergillus niger*.

The enzyme is intended for use as a processing aid in the production of brewed beverages. The proposed use is technologically justified at levels consistent with Good Manufacturing process (GMP). The enzyme preparation meets relevant identity and purity specifications in the Code.

T. reesei QM6a has a long history of safe use as a production microorganism of enzyme processing aids, including several that are already permitted in the Code. The production organism is neither pathogenic nor toxigenic. Analysis of the genetically modified production strain confirmed the presence and stability of the inserted DNA.

The amino acid sequence of the enzyme shows no homology with any known toxins, venoms or allergens, and the enzyme concentrate showed no genotoxic potential in a bacterial reverse mutation assay or a micronucleus assay conducted using human lymphocytes.

A No Observed Adverse Effect Level (NOAEL) of 1000 mg Total Organic Solids (TOS)/kg bw/day was identified in a 90-day oral toxicity study in rats. The theoretical maximum daily intake (TMDI) was calculated to be 0.31 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 3200.

In the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate for this prolyl oligopeptidase.

Table of contents

1 Introduction

IFF Australia Pty Ltd, trading as Danisco Australia Pty Ltd (the applicant) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme prolyl oligopeptidase, EC 3.4.21.26. This prolyl oligopeptidase is produced from a genetically modified (GM) strain of *Trichoderma reesei* containing the prolyl oligopeptidase gene from *Aspergillus niger*. The specific name for the production strain used by the applicant is confidential commercial information.

The enzyme is intended to be used as a processing aid in the production of brewed beverages. The stated purpose is to prevent chill haze caused by proline/glutamate rich proteins and peptides. The usage level is 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 a solely technological purpose 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 food enzyme produced by a GM microorganism, by considering the:
  - safety and history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s)
  - safety and history of use of the production organism
  - safety of the enzyme.

[1] GMP is defined in section 1.1.2—2 of the Code as follows: *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.

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 et al. 2009).

IUBMB name:	Prolyl oligopeptidase
Systematic name:	Prolyl oligopeptidase
Other names/common names:	Post-proline cleaving enzyme, proline-specific endopeptidase, postproline endopeptidase, proline endopeptidase, endoprolylpeptidase, prolyl endopeptidase
IUBMB enzyme nomenclature:	EC 3.4.21.26
CAS number:	72162-84-6
Reaction:	Prolyl oligopeptidase catalyses the hydrolysis of proline (pro^+) and alanine (ala^+) in oligopeptides

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 2020).

All inputs used in the production of the enzyme preparation are specifically intended for use in the production of food enzymes.

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food. 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)), explicitly FAO/WHO (2006) and in the Food Chemicals Codex (FCC 2022), referenced in subsection 3—2 of Schedule 3 of the Code. Enzymes used as a processing aid need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3. In addition, enzyme preparations must meet the JECFA specifications contained in the individual monographs where they exist. In the case of prolyl oligopeptidase, there is no individual monograph.[3]

[2] International Union of Biochemistry and Molecular Biology.
[3] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/.

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.

The applicant provided the results of analysis of two different batches of their prolyl oligopeptidase. Table 1 provides a comparison of the results of those analyses with specifications established by JECFA and Food Chemicals Codex, as well as those in the Code where applicable. Based on those results, the enzyme met all relevant specifications.

Table 1 Analysis of manufacturer's final enzyme preparation (prolyl oligopeptidase produced from a GM strain of* Trichoderma reesei*) compared to JECFA, Food Chemicals Codex and Code specifications for enzymes

Test parameters	*IFF Pty Ltd test results*	*Specifications*		
		JECFA	*Food Chemicals Codex*	*The Code - section S3—4*
Lead (mg/kg)	<0.05, <0.5	≤5	≤5	≤2
Arsenic (mg/kg)	0.2, <0.5	-	-	≤1
Cadmium (mg/kg)	<0.01, <0.1	-	-	≤1
Mercury (mg/kg)	<0.01	-	-	≤1
Coliforms (cfu*/g)	<1	≤30	≤30	-
***Salmonella* (in 25 g)**	Negative	Absent	Negative	-
***Escherichia coli* (in 25 g)**	Negative	Absent	-	-
Antimicrobial activity	Negative	Absent	-	-

*cfu = colony forming units
Note: Where batch results are the same, only 1 is listed.

The absence of the production strain in the enzyme preparation has been confirmed by the manufacturer. Refer to Section 3.4 below for the total organic solids (TOS) value. TOS encompasses the enzyme component and other organic material from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients.

2.3 Technological purpose

Prolyl oligopeptidase is intended for use as a processing aid in the production of brewed beverages. The applicant confirmed use of the enzyme at GMP levels.

Prolyl oligopeptidase catalyses the hydrolysis of proline (pro^+) and alanine (ala^+) in oligopeptides. The applicant stated that the enzyme performs its technological function during the fermentation step in brewing, preventing chill haze caused by proline/glutamate rich proteins and peptides.

For a schematic representation of the hydrolysis reaction catalysed by prolyl oligopeptidase, refer to its record in the enzyme database BRENDA[4] .

[4] EC explorer - BRENDA Enzyme Database (brenda-enzymes.org)

Proline-specific peptidases, including prolyl oligopeptidase, are extensively used in food applications for proteins containing L-proline residues, such as gluten found in cereals like barley, malt, and wheat, which are used in brewing (Mika et al. 2015).

The technological purpose described by the applicant as preventing chill haze caused by proline/glutamate rich proteins and peptides is consistent with the typical function of prolyl oligopeptidase and is confirmed by general scientific literature. (Di Ghionno et al. 2017, Mika et al. 2015). The applicant stated related benefits for endopeptidase also include increased production capacity and optimised cold stabilisation processes.

The applicant provided information on the physical and chemical properties of their enzyme preparation, summarised in Table 2. The enzyme is heat-denatured at a temperature of 75°C, therefore it is deactivated during the brewing process and no technological function in the final food.

Table 2 Prolyl oligopeptidase enzyme preparation physical/chemical properties

Physical/chemical properties of commercial enzyme preparation	
Enzyme activity	≥6.0 PEPU/g[5]
Appearance	Liquid, amber to brown in colour
Temperature range	Optimum 40-60°C
Temperature stability	The enzyme is completely deactivated after 30 minutes at temperatures above 75°C.
pH range and optimum	4 – 5.5.

2.4 Allergen considerations

The applicant has stated that a wheat-based material is used during fermentation and is wholly consumed during fermentation. The Product Data Sheet for the enzyme preparation indicated an absence of cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, khorasan wheat).

2.5 Food technology conclusion

FSANZ concludes that using this prolyl oligopeptidase as a processing aid in the production of brewed beverages is consistent with its typical function of catalysing the hydrolysis of proline (pro+) and alanine (ala+) in oligopeptides. The evidence provided supports its proposed use, ensuring that the enzyme, when used in the specified quantity and form (consistent with GMP), is technologically justified and effective in achieving its intended purpose.

Prolyl oligopeptidase fulfils its technological role during the production of brewed beverages. After this process, the enzyme is inactivated and does not serve any technological purpose in the final product. It is therefore, functioning as a processing aid for the purposes of the Code.

[5] The activity of the prolyl oligopeptidase is defined as ‘Proline Specific Endoprotease Units (PEPU) per gram.

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

3.1 Source microorganism

The Code currently permits other processing aids derived from *T. reesei* QM6a. 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 (US EPA 2012).

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

Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of enzymes for a range of industries including food (Nevalainen and Peterson, 2014; Paloheimo et al. 2016). *T. reesei* QM6a strains have a long history of safe use for commercial production of enzymes.

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 (US EPA 2012). 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 (US EPA 2012).

T. reesei is listed as Risk Group 1 in the microorganism classification lists of the German Federal Institute for Occupational Safety and Health (BAuA 2016) and meets the requirements of a Biosafety Level 1 organism based on the Biosafety in Microbiological and Biomedical Laboratories guidelines (US CDCP and NIH 2020).

FSANZ has identified no microbiological safety concerns related to T. reesei QM6a or its derivatives as a source organism for processing aids provided that requirements for genetic modifications are met and appropriate production methodology and controls are applied.

Data provided by the applicant confirmed the identity of the production strain as T. reesei. The production strain was developed by inactivating genes and integration of the desired genes into the host genome. The production organism has been genetically modified.

Data was provided by the applicant to demonstrate that this fungal strain does not produce toxicologically significant amounts of mycotoxins. The source organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production.

The analysis of characteristics of representative batches of enzyme along with the described production methodology demonstrated that culture conditions can be applied appropriately

and consistently between batches. Results confirming the absence of the production organism in the final enzyme production were provided by the applicant.

No public health and safety concerns were identified, and the production organism is neither pathogenic nor toxigenic.

3.2 Characterisation of the genetic modification

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

The gene that encodes the prolyl oligopeptidase enzyme was codon optimised and chemically synthesised based on the sequence from *Aspergillus niger* available in public databases. Data provided by the applicant and analysed by FSANZ confirmed the identity of the prolyl oligopeptidase enzyme.

Two expression cassettes containing the prolyl oligopeptidase gene were inserted into the genome of the host strain using standard molecular biology techniques. The prolyl oligopeptidase gene was placed under the control of the native *T. reesei* protein signal, *cbhl* gene promoter and terminator sequences. The expression cassettes were integrated at specific integration sites in the host's genome. The final production strain was selected based on high prolyl oligopeptidase activity.

3.2.2 Characterisation of inserted DNA

Whole genome sequencing (WGS) data provided by the applicant confirmed the presence of the inserted DNA in the production strain. No antibiotic-resistance markers are present in the final production strain.

3.2.3 Genetic stability of the inserted gene

The stability of the inserted DNA in the production strain was examined by WGS. DNA extracted from cultures after prolonged fermentation and stock culture prior to fermentation as a control were analysed. These data confirmed the prolyl oligopeptidase gene is expressed over multiple generations and is stable in the host's genome.

3.3 Safety of the prolyl oligopeptidase enzyme

3.3.1 History of safe use of the enzyme

This specific enzyme is approved for use in the United States of America and two countries in Europe. Further details were provided as confidential commercial information and considered as part of this assessment. However, evidence of the scope and duration of its commercial use is not available. There have been no reports of adverse effects attributed to its commercial use.

Demonstration of a history or safe use is not considered necessary for this application because toxicity data including a 90-day study in rats and genotoxicity assays have been provided.

3.3.2 Bioinformatics concerning homology with known toxins

BLAST searches were conducted against a recent (2023_05 of November 8, 2023) version of the UniProt annotated Protein Knowledge database[6]. None of the top 1000 matches in a

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

BLAST search for homology of the enzyme sequence against the complete UniProt database, with a threshold E-value of 0.1, was annotated as either a toxin or a venom. The majority of matches were peptidases. A BLAST search was also performed against the UniProt animal toxin database. This search also yielded no matches.

3.3.3 Toxicology data

Toxicology data submitted in support of the application include a 90-day oral gavage study in rats, a bacterial reverse mutation assay, and a mammalian cell micronucleus assay performed *in vitro* using human peripheral blood lymphocytes. The studies are summarized in Appendix 1. All three studies were performed in accordance with relevant OECD test guidelines, and using the same batch of prolyl oligopeptidase, which is representative of the commercial enzyme, as the test article.

No test article-related effects were observed in the Sprague Dawley rats that comprised the test system in the 90-day study. The No Observed Adverse Effect Level (NOAEL) was therefore identified as the highest dose level, which was 1000 mg TOS/kg bw/day.

No evidence of mutagenicity was observed in the bacterial reverse mutation assay, with or without the presence of S9 mix for metabolic activation, at concentrations up to and including 5000 µg/plate. Appropriate positive control articles were tested in parallel and induced more than threefold increases in revertant colonies when compared to negative control plates, confirming the validity of the assay.

No increase in the incidence of micronuclei was observed in cultured human peripheral blood lymphocytes exposed to prolyl oligopeptidase at concentrations up to and including 5000 µg/mL, either in the presence or absence of S9 mix for metabolic activation.

3.3.4 Potential for allergenicity

The application included reports of results of sequence homology searches conducted using the AllergenOnline[7] database version 22 (25 May 2023). The searches included a full-length sequence alignment, a sliding window of 80-amino acid sequences derived from the full-length amino acid sequence, and a search for an exact 8 amino acid match. No matches to known allergens were identified by any of the three search strategies.

3.3.5 Assessments by other regulatory agencies

There are no safety assessments by other regulatory authorities available for this specific enzyme.

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 ‘worst-case scenario’ approach to estimating likely levels of dietary exposure, assuming that all the TOS from the prolyl oligopeptidase 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

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

(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 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 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.
- All non-milk beverages contain the highest use level of 12.33 mg TOS/kg in the raw material (wort).
- The ratio of raw material (wort) to final food (beer, alcoholic beverage, non-alcoholic cereal based beverage) is 1.
- Final food containing the theoretical amount of TOS is consumed daily over the course of a lifetime.
- The enzyme preparation is not added to any solid foods.
- All of the TOS from the enzyme preparation remains in the final food.

These assumptions were deemed to be conservative and reflective of a first tier in estimating dietary exposure. Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.31 mg TOS/kg body weight/day.

An independent calculation of the TMDI was not undertaken by FSANZ as FSANZ agreed with the inputs and assumptions made by the applicant.

The applicant's estimate of the TMDI is an overestimate 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 beverages whereas the applicant has stated that it is likely to either be reduced or removed during processing, or would be present in insignificant quantities. In addition, the enzyme would be inactivated and perform no function in the final food to which the ingredient is added.

4 Discussion and conclusion

No public health and safety concerns were identified concerning the use of the production organism, which is neither pathogenic nor toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

The amino acid sequence of the enzyme shows no homology with any known toxins, venoms or allergens, and the enzyme concentrate showed no genotoxic potential in a bacterial reverse mutation assay or a micronucleus assay conducted using human lymphocytes.

A NOAEL of 1000 mg TOS/kg bw/day was identified in a 90-day oral toxicity study in rats. The theoretical maximum daily intake (TMDI) was calculated to be 0.31 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 3200.

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.

5 References

Adgyl Lifesciences Private Limited (2023a). DP-Nyq99: Repeated Dose (90-day) Oral Toxicity Study by Oral Gavage in CD Sprague Dawley Rats. Unpublished study report

Adgyl Lifesciences Private Limited (2023b). DP-Nyq99: Bacterial Reverse Mutation Test. Unpublished study report

Adgyl LifeSciences Private Limited (2023c). DP-Nyq99: *In vitro* Mammalian Cell Micronucleus Test in Human Peripheral Blood Lymphocyte. Unpublished study report.

Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA) (2016). Technische Regel für Biologische Arbeitsstoffe 460. https://www.baua.de/DE/Angebote/Rechtstexte-und-Technische-Regeln/Regelwerk/TRBA/TRBA-460.html. Accessed 16 September 2024

Di Ghionno L, Marconi O, Sileoni V, De Francesco G, Perretti G. (2017) Brewing with prolyl endopeptidase from *Aspergillus niger*: the impact of enzymatic treatment on gluten levels, quality attributes and sensory profile. International Journal of Food Science and Technology 52(6): 1367-1374

Douglass JS, Barraj LM, Tennant DR, Long WR, Chaisson CF (1997) Evaluation of the Budget Method for screening food additive intakes, Food Additives and Contaminants 14:791-802

FAO/WHO (2009) Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food Chapter 6 – Dietary exposure assessment of chemicals in food, WHO, Geneva

FAO/WHO (2020) Environmental Health Criteria 240. Principles and Methods for the Risk Assessment of Chemicals in Food. Chapter 6: Dietary exposure assessment of chemicals in food. Second Edition 2020. WHO, Geneva. https://www.who.int/docs/default-source/food-safety/publications/chapter6-dietary-exposure.pdf?sfvrsn=26d37b15_6

FAO/WHO (2021) Evaluation of certain food additives: eighty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series No. 1027.

Frisvad JC, Møller LL, Larsen TO, Kumar R, Arnau J (2018) Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*. Applied Microbiology and Biotechnology 102(22): 9481-9515

Kubicek CP, Komoń-Zelazowska M, Sándor E, Druzhinina IS (2007) Facts and Challenges in the Understanding of Biosynthesis of Peptaibols by Trichoderma. Chemistry and Biodiversity 4(6):1068-1082

Mika N, Zorn H, Rühl M. (2015) Prolyl-specific peptidases for applications in food protein hydrolysis. Applied Microbiology and Biotechnology 99: 7837–7846

Nevalainen H and Peterson R (2014) Making recombinant proteins in filamentous fungi - are we expecting too much? Frontiers of Microbiology 5:e75

Nevalinen H, Suominen P, Taimisto K (1994) Minireview. On the Safety of *Trichoderma reesei* Journal of Biotechnology 37:193-200

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms – A review. Regulatory Toxicology and Pharmacology 45:144-158

Paloheimo M, Haarmann T, Mäkinen S, Vehmaanperä J (2016) Production of industrial enzymes in *Trichoderma reesei*. In: Schmoll M, Dattenböck C (Eds) Gene expression systems in Fungi: Advancements and Applications, Springer International Publishing, Switzerland, p.23-57

Peterson R and Nevalainen H (2012) *Trichoderma reesei* RUT-C30-thirty years of strain improvement. Microbiology 158: 58-68

US EPA (2012) 40 CFR Part 725 Microorganisms; General Exemptions From Reporting Requirements; Revisions to Recipient Organisms Eligible for Tier I and Tier II Exemptions https://www.federalregister.gov/documents/2020/03/10/2020-04746/microorganisms-general-exemptions-from-reporting-requirements-revisions-to-recipient-organisms. Accessed 16 September 2024

US CDCP and NIH (2020) Biosafety in Microbiological and Biomedical Laboratories https://www.cdc.gov/labs/pdf/SF__19_308133-A_BMBL6_00-BOOK-WEB-final-3.pdf. Accessed 16 September 2024

Appendix 1: Summary of toxicology studies

Ninety-day oral gavage study of prolyl oligopeptidase enzyme concentrate in Sprague Dawley rats (Adgyl Lifesciences Pvte. Ltd 2023a). Regulatory status: GLP, conducted in compliance with OECD Guideline 408

The test article for this study was prolyl oligopeptidase concentrate with a Total Organic Solids (TOS) proportion of 27.94%. The vehicle and negative control article was deionised (MilliQ) water. Stability of the test article in the vehicle was determined prior to the study, and dose formulations from Days 1, 48 and 78 were analysed to confirm concentrations. Rats, 10/sex/group, were acclimatised to standard laboratory environmental and husbandry conditions prior to study start, at which they were aged 6 to 7 weeks. Rats were pair-housed by sex and administered 0, 250, 500, or 1000 mg TOS/kg bw/day by oral gavage, at a constant dose volume of 3.28 mL/kg bw/day. Observations and parameters recorded during the study included survival, clinical observations, bodyweights and bodyweight gain, feed consumption, results of ophthalmological assessments (prestudy and prior to scheduled termination), and results of neurobehavioural assessments (Days 83 and 84). Blood and urine were collected for analysis immediately prior to scheduled termination. Vaginal smears were collected from female rats after death, and all rats were subject to detailed necropsy. A standard list of fresh organ weights were recorded and a comprehensive list of organs and tissues were preserved for histopathological examination.

Dose analyses confirmed that the dose formulations were within an acceptable range of the target doses. All animals survived to scheduled termination and there were no treatment-related effects on clinical observations, bodyweights, bodyweight changes, feed consumption, ophthalmological findings, results of neurological assessments, haematology, clinical chemistry, coagulation parameters, urine parameters, thyroid hormone measurements, oestrous cycles, organ weights, findings on gross necropsy or findings on histological examination. The No Observed Adverse Effect Level was therefore identified as the highest dose used in the study, 1000 mg TOS/kg bw/day.

Bacterial reverse mutation assay of prolyl oligopeptidase concentrate (Adgyl Lifesciences Pvte. Ltd 2023b). Regulatory status: GLP, conducted in compliance with OECD Guideline 471, as corrected June 2020.

Sterile water was used as the vehicle and the negative control article. Positive controls were dissolved in DMSO. The test strains were *Salmonella enterica* Var. Typhimurium histidine auxotrophic strains TA98, TA 100, TA1535 and TA 1537, and the tryptophan auxotrophic strain of *Escherichia coli* WP2*uvr*A. The assays were conducted by the treat-and-plate method with the exception of the S9 positive control for *Escherichia coli*, for which the plate incorporation method was used.

In an initial toxicity assay, the TA100 strain and the vehicle control were exposed to 0, 50, 100, 200, 400, 800, 1600, 3200 and 5000 µg total organic solids (TOS)/plate test doses. Assays were conducted in duplicate, in the presence and absence of S9 mix for metabolic activation. Plates were examined for revertant colonies, bacterial lawn and evidence of precipitation.

In the absence of any evidence of cytotoxicity or precipitation of the test article in the toxicity assay, the concentrations selected for the subsequent assays were 0, 50, 158, 500, 1581 and 5000 µg/plate. All the test strains were used in the assays, which were conducted in

triplicate, with or without the addition of S9 mix for bacterial activation. Initial and confirmatory assays were conducted. No increase in the mean number of revertant colonies was observed for any tester strain when compared to the respective vehicle control plates, either in the presence or absence of S9 mix. Positive control chemicals tested concurrently produced a greater than threefold increase in the mean numbers of revertant colonies in all the test strains when compared to the respective vehicle control plates, confirming the validity of the assay.

It was concluded that the test article was not mutagenic at dose levels up to and including a dose level of 5000 µg/plate, which is the highest dose recommended in OECD test guideline 471, under the conditions of the assay.

In vitro *mammalian cell micronucleus test of prolyl oligopeptidase concentrate in human peripheral blood lymphocytes (Adgyl Lifesciences Pvte. Ltd 2023c). Regulatory status: GLP, conducted in compliance with OECD Guideline 487.*

The study consisted of a preliminary assay to determine cytotoxicity, followed by the micronucleus assay. The test system was human lymphocytes in whole blood culture, stimulated to divide by addition of phytohaemagglutinin 48 hours prior to treatment. The lymphocytes were exposed to the test item in the presence and absence of S9 mix as a metabolic activation system. Prior to the tests, the test article was determined to be soluble in sterile water at concentrations ≤ 50 mg/mL.

In the preliminary cytotoxicity test, blood cultures were exposed to the test article at concentrations up to 5000 µg/mL. The test item did not show precipitation in the test medium in any of the tests, with or without S9 mix, and did not cause any appreciable change in the pH or osmolality of the treatment medium at any of the tested concentrations compared to the sterile water control. A moderate (29 to 36%) reduction in cytokinesis block proliferative index (CBPI) occurred at the highest tested concentration of 5000 µg/mL under all of three test conditions, when compared to the water control.

The micronucleus assay was carried out in duplicate. Blood cultures were exposed to the test article at concentrations of 0, 312.5, 1250 and 5000 µg/mL in each of three independent experiments. Experiments 1 and 2 comprised a 3 h exposure in the presence and absence of metabolic activation, respectively, and Experiment 3 comprised a 24 h exposure in the absence of metabolic activation. Vehicle control assays and assays with appropriate positive control articles were run concurrently in duplicate.

Cytokinesis was blocked following mitosis using Cytochalasin B and each culture from the controls and treatment levels was harvested approximately 24 hours after the beginning of the treatment. The frequencies of micronuclei from 2000 bi-nucleated cells per concentration were analyzed. There was no evidence of statistically significant induction of micronuclei, in the presence or in the absence of metabolic activation, at any of the tested concentrations of the test article. Under identical conditions, the respective positive control substances produced large, statistically significant increases in micronuclei, confirming the validity of the assay.

It was concluded that the test article did not induce micronuclei in cultured human peripheral blood lymphocytes, either in the presence or in the absence of metabolic activation, at ≤ 5000 µg/mL under the conditions of the assay.