A Prolyl oligopeptidase Enzyme
from a recombinant strain of *Trichoderma reesei*

PROCESSING AID APPLICATION

Food Standards Australia
New Zealand

Applicant: IFF AUSTRALIA PTY LTD (Trading as Danisco Australia Pty Ltd)

8th August 2024

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EXECUTIVE SUMMARY:

IFF is seeking approval for a "Prolyl oligopeptidase (EC 3.4.21.26)" enzyme for use as processing aid in brewing application. The enzyme is designated as "Prolyl oligopeptidase" throughout the dossier.

The enzyme Prolyl oligopeptidase is derived from a selected non-pathogenic, non-toxigenic strain of *Trichoderma reesei* which is genetically modified to express the Prolyl oligopeptidase gene from *Aspergillus niger.*

The enzyme is intended for use in the production of brewed beverages. In brewing, Prolyl oligopeptidase performs is function typically during the fermentation step of the brewing process.

In all of these applications, Prolyl oligopeptidase will be used as a processing aid where 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.

To assess the safety of the Prolyl oligopeptidase for use in these applications, IFF vigorously applied the criteria identified in the guidelines as laid down by Food Standards Australia New Zealand (FSANZ) and U.S. Food and Drug Administration (FDA) utilising enzyme toxicology/safety data, the safe history of use of enzyme preparations from *T. reesei* and of other enzymes in food, the history of safe use of the *T. reesei* production organism for the production of enzymes used in food, an allergenicity evaluation, and a comprehensive survey of the scientific literature.

In addition, different endpoints of toxicity were investigated, and the results are evaluated and assessed in this document. In genotoxicity studies, Prolyl oligopeptidase is not mutagenic, clastogenic or aneugenic. Daily oral administration of Prolyl oligopeptidase up to and including a dose level of 1000 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Based on a worst-case scenario that a person is consuming Prolyl oligopeptidase from a brewed beverage, the calculated Theoretical Maximum Daily Intake (TMDI) will be 0.31 mg TOS/kg body weight/day. This still offers a 3226-fold margin of safety.

Based on the results of safety studies and other evidence, Prolyl oligopeptidase has been demonstrated as safe for its intended applications and at the proposed usage levels. Approval of this application would provide manufacturers and/or consumers with benefits of facilitating the brewing process.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Danisco Australia Pty Ltd

(b) Company:

Danisco Australia Pty Ltd

(c) Address:
Ground floor, 97 Waterloo Rd
Macquarie Park
NSW 2113
Australia

(d) Contact Details:

[redacted]
Regulatory Affairs Manager

Tel : [redacted]
Email: [redacted]

(e) Email address:

See above

(f) Nature of Applicants Business:
Danisco Australia Pty Ltd – A subsidiary of International Flavors and Fragrances Inc (IFF), manufacturer/marketer of specialty food ingredients, food additives and food processing aids.

(g) Details of Other Individuals etc.:

No other individuals, companies or organisations are associated with this application.

1.2 Purpose of the application

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application. The intended use of the processing aid is in brewed beverages.

This application is made solely on behalf of IFF, the manufacturer/marketer of the Processing Aid. When approved, the Processing Aid would be available for use by any food manufacturer in Australia and New Zealand.

Prolyl oligopeptidase, subject of this application, is intended for use in alcoholic and non-alcoholic brewed beverages.

Currently no prolyl oligopeptidase from *Aspergillus niger* expressed in *Trichoderma reesei* is permitted as a Processing Aid, however Xylanase from *T. reesei*, and other enzymes including Cellulase, Endo-1,4-beta-xylanase, β-Glucanase, Hemicellulase multicomponent enzyme, Polygalacturonase or Pectinase multicomponent enzyme, from *T. reesei* are listed in Schedule 18 section S18-4(5) as permitted enzymes. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in Section 2.3 and Appendix A.

1.3 Justification for the application

1.3.1. Regulatory Impact Information

A. Costs and Benefits of the application
Prolyl oligopeptidase is an enzyme produced by submerged fermentation of *T. reesei* carrying the gene encoding the prolyl oligopeptidase from *A. niger.* The enzyme is characterised as a proline-specific endopeptidase (EC 3.4.21.26). A collection of information detailed in Section 3 supports the safety of the production organism and the enzyme for use in the applications outlined in Section 4.

The enzyme is intended for use in the brewing for the production brewed beverages. In brewing, Prolyl oligopeptidase performs its technological function performs is function typically during the fermentation step of the brewing process for the primary purpose of preventing chill haze caused by proline/glutamate rich proteins and peptides.

More information on the benefit of this enzyme can be found in Section 2.2 and Appendix A.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no prolyl oligopeptidase from *A. niger* expressed in *T. reesei* is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

B. Impact on international trade
The inclusion of Prolyl oligopeptidase from *A. niger* expressed in *T. reesei* in the Australia New Zealand Food Standards Code as a processing aid may promote international trade on products produced with this enzyme product and reduce technical barriers to trade.

1.4. Support for the application

No marketing or promotional activities have been undertaken for Prolyl oligopeptidase derived from *T. reesei* containing the gene for Prolyl oligopeptidase from *A. niger* in the Australia/New Zealand market. Hence at this stage, no requests from food manufacturers are provided in support of this application. However, the need and justification for use of the processing aid are discussed in Section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

1.5. Assessment Procedure

This application seeks to modify Schedule 18 to Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Based on guidance in the Application Handbook, IFF considers General Procedure Level 1 (up to 350 hours) to be the appropriate procedure for assessment of the application.

1.6. Confidential Commercial Information (CCI)

Certain (identified) technical and manufacturing information included in Appendices A2, A10, Appendices B1, B3-B5, B7-B8, B10-B11, Appendix D1-D3, and Appendices E1-E5 labelled with 'Confidential Commercial Information', is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

Certain redactions throughout the dossier have also been made to avoid identification or disclosure of proprietary strain, product or other details considered commercially sensitive. Both redacted and non-redacted versions of all documentation have been supplied to FSANZ. The applicant requests that only redacted versions are provided for public consultation purposes.

1.7. Exclusive Commercial Capturable Benefit (ECCB)

According to Section 8 of the FSANZ Act, this application is not expected to confer Exclusive Capturable Commercial Benefit (ECCB).

1.8. International and other National Standards

Refer to Appendix D for further details.

1.8.1 Codex Standards

Prolyl oligopeptidase from *A. niger* expressed in *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

1.8.2 International Legislation

[redacted]

1.9. Statutory declaration

I, [redacted]

of [redacted] Regulatory Affairs Manager/Director

make the following declaration under the Oaths and Declaration Act 1959:

the information provided in this application fully sets out the matters required; and
the information is true to the best of my knowledge and belief; and
no information has been withheld which might prejudice this application to the best of my knowledge and belief.

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, and I believe that the statements in this declaration are true in every particular.

Signature [redacted]

Declared at Auckland on 26 of July 2024

Before me, [redacted]

[redacted]
Lawyer
Kemps Weir Lawyers Limited
Auckland

Signature [redacted]

1.10. Checklist

	Mandatory Requirements	Check	Page Number	Remarks
General requirements for applications	A. Form of the application	✓	N.A.	
	Table of contents	✓	1	
	Executive summary	✓	2	
	B. Applicant details	✓	3	Section 1.1
	C. Purpose of application	✓	4	Section 1.2
	D. Justification for the application	✓	4	Section 1.3
	D.1 Regulatory impact information	✓	4	Section 1.3.1
	D.1.1 Costs and benefits of the application	✓	4	Section 1.3.1
	D.1.2 Impact on international trade	✓	4	Section 1.3.1
	E Information to support the application	✓	5	Section 1.4
	E.1 Data requirements	✓	N.A.	
	F. Assessment procedure	✓	5	Section 1.5
	G. Confidential commercial information (CCI)	✓	5	Section 1.6
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	I. Exclusive capturable commercial benefit (ECCB)	✓	5	Section 1.7
	J. International and other national standards	✓	5	Section 1.8
	J.1 International Standards	✓	5	Section 1.8.1
	J.2 Other national standards or regulations	✓	5	Section 1.8.2
	K. Statutory declaration	✓	6	Section 1.9
	L. Checklist	✓	7	Section 1.10
3.3.2. Processing aids	A. Technical information on the processing aid	✓	9	Section 2
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	A.2 Information on the identity of the processing aid	✓	9	Section 2.2
	A.3 Information on the chemical and physical properties of the processing aid	✓	9	Section 2.3
	A.4 Manufacturing process	✓	10	Section 2.4
	A.5 Specification for identity and purity	✓	11	Section 2.5
	A.6 Analytical method for detection	✗		Not applicable for enzymes used as processing aids
	C. Information related to the safety of an enzyme processing aid	✓	12	Section 3
	C.1 General information on the use of the enzyme as a food processing aid in other countries	✓	12	Section 3.1
	C.2 Information on the potential toxicity of the enzyme processing aid	✓	13	Section 3.2
	C.3 Information on the potential allergenicity of the enzyme processing aid	✓	14	Section 3.3

	C.4 Safety assessment reports prepared by international agencies or other national government agencies, if available	✓	14	Section 3.4
	D. Additional information related to the safety of an enzyme processing aid derived from a microorganism		14	Section 3
	D.1 Information on the source microorganism	✓	14	Section 3.5
	D.2 Information on the pathogenicity and toxicity of the source microorganism	✓	14	Section 3.6
	D.3 Information on the genetic stability of the source organism	✓	15	Section 3.7
	E. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism			Section 3
	E.1 Information on the methods used in the genetic modification of the source organism	✓	15	Section 3.8
	F Information related to the dietary exposure to the processing aid		16	Section 4
	F.1. A list of foods or food groups likely to contain the processing aid or its metabolites	✓	16	Section 4.1
	F.2 The levels of residues of the processing aid or its metabolites for each food or food group	✓	16	Section 4.2
	F.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption	✓	17	Section 4.3
	F.4 The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid	✓	17	Section 4.4
	F.5 Information relating to the levels of residues in foods in other countries	✓	17	Section 4.5
	F.6 For foods where consumption has changed in recent years, information on likely current food consumption	✓	17	Section 4.6

2. Technical information

Please refer to Appendix A for further details

2.1. Type of processing aid

The Prolyl oligopeptidase enzyme is an enzyme produced by submerged fermentation of *T. reesei*, carrying the proline specific endopeptidase gene from *A. niger*.

This Processing Aid falls into the category "Enzymes of microbial origin" from the Food Standard Code section 1.3.3-6 Enzymes.

2.2. Identity

2.2.1 Chemical/Common Name:
The systematic name of the principal enzyme activity is prolyl oligopeptidase (proline specific endopeptidase). Other names used are post-proline cleaving enzyme; proline-specific endopeptidase; post-proline endopeptidase; proline endopeptidase; endoprolylpeptidase; prolyl endopeptidase.

- EC number: 3.4.21.26
- CAS number: 72162-84-6

Biological source: The Prolyl oligopeptidase enzyme is an enzyme produced by submerged fermentation of *Trichoderma reesei*, carrying the proline specific endopeptidase gene from *Aspergillus*.

2.2.2 Marketing Name of the Processing Aid:
The marketing name of this enzyme preparation will depend on the application. An example marketing name of Prolyl oligopeptidase is [redacted]

2.2.3 Molecular and Structural Formula:
Prolyl oligopeptidase is a protein. The amino acid sequence is known. Please refer to Appendix E (Confidential Commercial Information).

2.3. Chemical and physical properties

The function of Prolyl oligopeptidase is to catalyse the hydrolysis of the hydrolysis of pro+ and ala+ in oligopeptides. When added to the brewing process during fermentation under controlled conditions, Prolyl oligopeptidase cleaves the naturally occurring peptide substrates in the brew to deliver the below benefits:

- Prevents chill haze caused by proline/glutamate rich proteins and peptides
- Increases production capacity.
- Optimised cold stabilisation procedure.
- Reduced sustainability footprint (energy & water) and applicable for a broad range of brewing
- protocols and process conditions
- Ideal solution for membrane filtration process

Substrate specificity:

The function of Prolyl oligopeptidase is to catalyse the hydrolysis of pro+ and ala+ in oligopeptides. In principle, the enzymatic conversion of proteins/oligopeptides with the help of

prolyl oligopeptidase may be used in the processing of all food raw materials which naturally contain these substrates.

Activity:
The activity of the Prolyl oligopeptidase is defined in PEPU. The assay is based on the ability of proline specific endo-protease to cleave p-nitroanalide from the synthetic substrate Z-Gly-Pro-pNA to form a colorimetric reaction that can be read at 405nm on an automated analyser. The increase in absorbance is related directly to enzyme activity via the use of an enzyme standard

pH and temperature profile:
The activity of the Prolyl oligopeptidase from *Trichoderma reesei* was measured under various pH and temperature conditions. The results (see Appendix A) show the pH optimum is 4-5.5, The data show that Prolyl oligopeptidase activity declines after at temperatures greater than 60˚C when incubated for 30 minutes. The enzyme is deactivated at 75˚C.

Interaction of the enzyme with different foods:
The Prolyl oligopeptidase enzyme preparation will be used as a processing aid where the enzyme is not present or active in the final food or present in negligible amounts with no technical function in the final food.

Nutritional implication:
Prolyl oligopeptidase is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of Prolyl oligopeptidase e are very low, and as with other enzymes that are currently approved and used as Processing Aids, use of this preparation would not have any nutritional significance.

2.4. Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are provided in Appendix E. Note that this information is proprietary and “Confidential Commercial Information” status is requested.

The production of Prolyl oligopeptidase is monitored and controlled by analytical and quality assurance procedures that ensure that the finished preparation complies with the specifications and is of the appropriate quality for use as a processing aid in food processing applications.

2.5. Specification for identity and purity

Impurity profile:

Appropriate GMP controls and processes are used in the manufacture of Prolyl oligopeptidase to ensure that the finished preparation does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits are as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 10,000 CFU/g
Total coliforms	less than 30 CFU/g
E. coli	absent in 25g
Salmonella	absent in 25g
Antibiotic activity	negative by test
Physical properties:	
Appearance	amber to brown liquid

Standard for identity:

Prolyl oligopeptidase meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

3. Safety

Refer to Appendix B for further details

3.1. Use of the enzyme as a food processing aid in other countries

Enzyme products are developed for a specific function, i.e., to catalyse a specific chemical reaction. That reaction determines the IUBMB classification. Enzyme variants may be selected to have a better performance of that function under the specific conditions of the application (e.g., temperature or pH). Enzymes of a certain IUBMB classification share conserved structural elements, called domains, which are needed for their specific function. As such the enzymes of our approval procedures do resemble those already permitted by FSANZ both in function and in structure.

Figure 1 below shows an example of natural variation of alpha-amylases. The same holds for any other enzyme type. While significant differences in sequence amongst the various species exist, they all catalyse the same reaction and therefore fit under the same IUBMB entry. There will also be natural variation within one species. All this also applies to the enzymes under the current approval procedures by FSANZ:

% amino acid sequence identity	*B. amyloliquefaciens*	*B. licheniformis*	*G. stearothermophilus*	*A. niger*	*A. oryzae*	*Z. mays*	*O. sativa*	*H. vulgare*	*P. vulgaris*	*H. sapiens*
Bacillus amyloliquefaciens	100									
Bacillus licheniformis	80	100								
Geobacillus stearothermophilus	65	65	100							
Aspergillus niger	21	21	22	100						
Aspergillus oryzae	23	24	24	66	100					
Zea mays (corn)	24	26	25	28	27	100				
Oryza sativa (rice)	25	27	25	27	26	89	100			
Hordeum vulgare (barley)	25	23	24	25	28	70	69	100		
Phaseolus vulgaris (bean)	26	27	25	24	27	67	65	64	100	
Homo sapiens (human)	25	33	29	22	28	23	22	23	24	100

α-amylases in nature have divergent

amino acid sequences but have the same catalytic activity and IUBMB number

Figure 1. Variation of enzymes in nature.

The expressed mature enzyme amino acid sequence of proline specific endopeptidase (the systematic name is prolyl oligopeptidase) shows a clear conserved abhydrolase superfamily domain, which includes substrate hydrolysis activities. The enzyme is also known as Endo-protease (EC 3.4.21.26).

Endo-protease enzyme, the subject of this dossier, is one of the permitted processing aids on Schedule 18 of the ANZ Food Standards Code, i.e., from *Aspergillus niger*. In our case the enzyme protein is expressed from *Trichoderma reesei*.

The endo-protease enzyme derived from *Trichoderma reesei* carrying the endo-protease gene from *Aspergillus niger* has been determined to be [redacted] There have not been any adverse events reported since this alpha-amylase has been in commercial use in these countries.

Please refer to section 1.8 and Appendix D for details on the different approvals in the countries listed above.

3.2. Toxicity of the enzyme

Toxin homology study
A BLAST search for homology of the aminopeptidase sequence against the complete Uniprot database (http://www.uniprot.org/), was performed, with a threshold E-value of 0.1. The majority of matches were peptidases, with none of the top 1000 database matches being annotated as either toxin or venom.

In addition, a specific BLAST search for homology of the mature aminopeptidase sequence was performed against the Uniprot animal toxin database. This yielded no matches. Therefore, the aminopeptidase sequence does not share homology with a known toxin or venom sequence. Refer to Appendix B for further information.

Safe Strain Lineage concept
The Safe Strain Lineage concept has been discussed by Pariza and Johnson (2001) in their publication on the safety of food enzymes and is commonly utilised by enzyme companies in the determination of the safety of their products for specific uses, as appropriate.

The primary issue in evaluating the safety of a production strain is its toxigenic potential, specifically the possible synthesis by the production strain of toxins that are active via the oral route. The toxigenic potential of the production organism is confined to the Total Organic Solid (TOS) originating from the fermentation.

As the toxicological evaluation is based on the TOS originating from fermentation of the production organism, studies conducted on strains from the Safe Strain Lineage can support other production strains pertaining to this same Safe Strain Lineage.

Although *T. reesei* is scientifically determined by IFF as a Safe Strain Lineage, the food enzyme object of the current dossier is supported by toxicological studies on the specific food enzyme object of this dossier. The toxicological studies on *T. reesei* [redacted] are thus one of the pillars supporting the IFF *T. reesei* Safe Strain Lineage. The position of the food enzyme in the IFF *Trichoderma reesei* Safe Strain Lineage is presented in Appendix B3 (Confidential Commercial Information).

Toxicological testing
To assess the safety of Prolyl oligopeptidase, different endpoints of toxicity were investigated and are evaluated and assessed in this document:

- Ames test: no mutagenic activity under the given test conditions
- Chromosomal aberrations: no clastogenic activity under the given test conditions
- 90-day oral toxicity on rats: the NOAEL (no observed adverse effect level) is established at the highest dose tested, 1000 mg total organic solid (TOS)/kg bw/day in male and female rats.

A summary of the results of the studies can be found in Appendix B. In addition, safety was further assessed according to the decision tree in the Pariza-Johnson guidelines (2001) for assuring the safety of a new enzyme preparation.

3.3. Allergenicity of the enzyme

Bioinformatic analyses based on sequence homology determined that the *A. niger* Prolyl oligopeptidase is unlikely to pose a risk of food allergenicity. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

An allergen statement is given in Appendix A9.

3.4. Safety assessment reports prepared by international agencies or other national government agencies

As discussed in section 1.8 Prolyl oligopeptidase from *A. niger* expressed in *T. reesei* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application. [redacted] Refer Appendix D for safety reports/approval letters (Confidential Commercial Information).

3.5. Information on the production microorganism

The production organism strain [redacted] is a strain of *T. reesei* which has been genetically modified by IFF to overexpress a prolyl oligopeptidase gene from *A. niger*.

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al.* (1994), Blumenthal (2004) and Olempska-Beer *et al.* (2006). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally recognised as a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is also considered as suitable for Good Industrial Large Scale Practice (GILSP) worldwide and meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001). There were 3 expression cassettes of the prolyl oligopeptidase gene integrated into the recipient genome.

Full details of the gene and recombinant microorganism are provided in Appendix E. Note that this information is proprietary and "Confidential Commercial Information" status is requested.

3.6. Pathogenicity and toxicity of the source microorgaism

T. reesei has a long history of safe use in industrial scale enzyme production. The safety of this species as an industrial enzyme producer has been reviewed by Nevalainen *et al.* (1994) and Blumenthal (2004). The organism is considered non-pathogenic for humans and does not produce fungal toxins or antibiotics under conditions used for enzyme production. It is generally considered a safe production organism and is the source organism of a range of enzyme preparations that are used as processing aids in the international food and feed industries. It is listed as a safe production organism for cellulases by Pariza and Johnson (2001) and Olempska-Beer *et al.* (2006), and various strains have been approved for the manufacture of commercial enzyme preparations by Food Standards Australia New Zealand, and internationally, for example, in Canada (Food and Drugs Act Division 16, Table V), the United States (21CFR § 184.1250), Mexico, Brazil, France, Denmark, China, and Japan. Further details are discussed in Appendix B.

3.7. Genetic stability of the production organism

The parental strain of the production strain *Trichoderma reesei* QM6a and its derivatives have been used for industry scale enzyme manufacturing for decades by IFF and its parental companies, and has demonstrated stable enzyme expression even at large scale fermentation. Please also refer to Appendix B3 (Confidential Commercial Information) for list of example enzyme preparations produced using QM6a and its derivatives. Furthermore, the production strain has demonstrated to be 100% stable as confirmed by genome sequencing.

3.8. Method used in the genetic modification of the source organism

The production organism of the Prolyl oligopeptidase preparation, the subject of this submission, is *T. reesei* strain [redacted]

It is derived by recombinant DNA methods from strain [redacted]. The purpose of this genetic modification is to enhance prolyl oligopeptidase production levels. [redacted], a commercial production strain, is derived, as a result of several classical mutagenesis steps, from the well-known wild-type strain QM6a. Virtually all strains used all over the world for industrial cellulase production today are derived from QM6a. The donor organism is *Aspergillus niger*. Prolyl oligopeptidase expression cassettes were integrated into the host genome. Full details of the genetic modifications are provided in Appendix E2 (Confidential Commercial Information).

The genetic stability of the inserted gene has been demonstrated by genome sequencing. NGS sequencing was used to characterise the production strain for the insertion site at generation 0 and compared to the end of fermentation. Any DNA rearrangement of the inserted expression cassettes was measured as to change of flanking DNA sequence in the analysis. No change was observed between the genomic DNA samples extracted from generation 0 to those extracted at the end of fermentation. This indicates there had been no insertions of the expression cassette at new sites in the *T. reesei*, indicating stability of the strain over the course of fermentation.

Full details of the genetic modifications and stability of the inserted genes are provided in Appendix E1-E3. Note that this information is proprietary and “Confidential Commercial Information” status is requested.

3. Dietary exposure

Refer to Appendix C for further details

4.1. List of food or food groups likely to contain the enzyme or its metabolites

According to the food group classification system used in Standard 1.3.1-Food Additives Schedule 15 (15-5), Prolyl oligopeptidase will be used in:

- 14.1 Non-alcoholic beverages and brewed soft drinks
- 14.2.1 Beer and related products

4.2. Levels of residues in food

The proposed application rate of Prolyl oligopeptidase in its intended application is listed below.

	Application	Raw Material (RM)	Maximal recommended use level (mg TOS/kg RM)	Example Final food (FF)	Rate RM/FF	Maximal level in FF (mg TOS/kg food)
Liquid Food	Brewing/ Alcoholic beverage/non-Alcoholic cereal based beverage	wort	12.33	Beer alcoholic Beverage non-alcoholic cereal based beverage	1	12.33

IFF expects the Prolyl oligopeptidase to be inactivated or removed during the subsequent production and refining processes for all applications.

Prolyl oligopeptidase performs its technological function during food processing. Like endogenous prolyl oligopeptidase present in raw materials and ingredients, it does not perform any technological function in the final food. The reasons why an enzyme does not exert any (unintentional) enzymatic activity in the final food can be due to a combination of various factors, depending on the application and the process conditions used by the individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, wrong pH, etc. In some cases (e.g. after alcohol distillation, products resulting from starch processing), an enzyme may no longer be present in the final food.

The most appropriate way to estimate the human consumption in the case of food enzymes is using the Budget Method (Hansen, 1966; Douglass *et al.*, 1997). This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data. The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

Based on the raw materials used in the various food processes, the recommended use levels of the enzyme Prolyl oligopeptidase, for the calculation of the TMDI, the maximum use levels are chosen. The TMDI is calculated on basis of the maximal values found in food and beverages multiplied by the average consumption of food and beverages per kg body weight/day. Consequently, the TMDI will be: 0.31 mg TOS/kg body weight/day. The NOAEL has been determined for Prolyl oligopeptidase to be at 1000 mg total protein/kg bw/day. Based on a worst-

case scenario of daily food consumption, the NOAEL would offer a 3226 fold margin of safety. It should be stressed that this Total TMDI is based on conservative assumptions and represents a highly exaggerated value. Please refer to Appendix C for details.

4.3. Likely level of consumption of foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs)

Not applicable. Prolyl oligopeptidase is not expected to be used in production of any foods or food groups that are currently not listed in NNSs. If such usage arises, an application would be made to inform FSANZ.

4.4. Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The enzyme would be used as a processing aid in about:

- 20% of brewed beverages sold in Australia and New Zealand

4.5. Levels of residues in food in other countries

Applications and levels of use of the Prolyl oligopeptidase preparation in other countries is the same as presented in section 4.2.

4.6. Likely current food consumption for foods where consumption has changed in recent years

Not applicable. Consumption of foods produced with Prolyl oligopeptidase is not expected to have a significant change.

5. References

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