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Supporting document 1

Risk and technical assessment – Application A1229

Carboxypeptidase from GM *Aspergillus oryzae* as a processing aid

Executive summary

Novozymes Australia Pty Ltd (Novozymes) submitted an application to permit the use of a carboxypeptidase (EC 3.4.16.6) from genetically modified (GM) *Aspergillus oryzae*, containing the carboxypeptidase gene from *A. oryzae*. The carboxypeptidase is proposed for use in the manufacture and/or processing of proteins, yeast and flavourings; the manufacture of bakery products; and brewing in accordance with Good Manufacturing Practice (GMP) conditions.

FSANZ has undertaken an assessment and concludes that the proposed use of carboxypeptidase as a processing aid in the manufacture and/or processing of proteins, yeast and flavourings; the manufacture of bakery products; and brewing is consistent with its known technological function to hydrolyse proteins into shorter proteins/peptides, and free amino acids by preferential release of a C-terminal arginine or lysine residue (BRENDA:EC3.4.16.6, 2022).

Carboxypeptidase performs its technological purpose during the production of food and is not performing a technological purpose in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code.

No public health and safety concerns were identified in the assessment of carboxypeptidase from GM *A. oryzae* under the proposed use conditions. *A. oryzae* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. The *A. oryzae* host is neither pathogenic nor toxigenic. Analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

The enzyme did not show significant homology with known toxins, or with known food allergens. Results of genotoxicity assays were negative. A no observed adverse effect level (NOAEL) of 2,220 mg total organic solids (TOS)/kg bodyweight (bw)/day was identified in a 90-day oral toxicity study in rats. The theoretical maximum daily intake (TMDI) of the TOS from the carboxypeptidase enzyme preparation was calculated to be 11.3 mg TOS/kg

bw/day. A comparison of the NOAEL and the TMDI results in a large Margin of Exposure (MOE) of approximately 200. 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. FSANZ concludes that there are no public health and safety concerns.

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1 Introduction

Novozymes Australia Pty Ltd (Novozymes) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme carboxypeptidase (EC 3.4.16.6) as a processing aid in the manufacture and/or processing of proteins, yeast and flavourings; the manufacture of bakery products; and brewing. This enzyme is sourced from a genetically modified (GM) strain of *Aspergillus oryzae* containing a carboxypeptidase gene from *A. oryzae*. The enzyme is marketed as a liquid preparation under the commercial name NS PP0083.

The application describes a newly developed enzyme. Therefore, this carboxypeptidase enzyme produced by GM *A. oryzae* containing the carboxypeptidase gene from *A. oryzae* needs pre-market assessment before permission can be given for its use as a processing aid. If permitted, the enzyme will provide an option for the manufacture and/or processing of proteins, yeast and flavourings; the manufacture of bakery products; and brewing.

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 (function) and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety concerns that may arise from the use of this enzyme, produced by a GM microorganism, as a processing aid, specifically by considering the:
 - history of use of the gene donor and production microorganisms
 - characterisation of the genetic modification(s), and
 - safety of the enzyme.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity and properties of the enzyme

The production microorganism of the enzyme is a GM strain of *A. oryzae*. The donor microorganism for the carboxypeptidase gene is *A. oryzae* (further details contained in Section 3). The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using the International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature database (IUBMB, 2018). Details of the identity of the enzyme are provided below.

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Accepted IUBMB name:	Carboxypeptidase D
Other names:	Cereal serine carboxypeptidase II; <i>Saccharomyces cerevisiae</i> KEX1 gene product; Carboxypeptidase Kex1; Gene KEX1 serine carboxypeptidase; KEX1 carboxypeptidase; KEX1 proteinase; KEX1DELTAp; CPDW-II; Serine carboxypeptidase (misleading); <i>Phaseolus</i> proteinase
IUBMB No.:	EC 3.4.16.6
CAS number:	153967-26-1
Reaction:	Preferential release of a C-terminal arginine or lysine residue

IUBMB: International Union of Biochemistry and Molecular Biology; CAS: Chemical Abstracts Service

2.2 Manufacturing process

2.2.1 Production of the enzyme

Novozymes' carboxypeptidase is produced by submerged fed-batch fermentation of *GM A. oryzae*. The main fermentation steps are inoculum, seed fermentation, and main fermentation. This is followed by the recovery stage which involves primary separation, germ filtration, and concentration to achieve the desired enzyme activity and/or to increase the ratio of enzyme activity to total organic solids (TOS) before formulation. The resulting product is a concentrated enzyme solution that the applicant states is free of the production strain and insoluble substances. The fermentation processes are consistent with the scientific literature and references provided with the application (Aunstrup 1979).

The application states that preparations are completed aseptically in accordance with Good Manufacturing Practices (GMP). Novozymes has provided certificates for compliance with ISO 9001:2015. Details of the manufacturing process, raw materials and ingredients used in the production of the carboxypeptidase enzyme preparation were provided in the application, some as Confidential Commercial Information (CCI).

The typical composition of the applicant's enzyme preparation is:

Enzyme solids (TOS)	approx. 4.0%
Sucrose	approx. 30.0%
Sodium chloride	approx. 10.0%
Potassium sorbate	approx. 0.2%
Water	approx. 55.8%

TOS: Total organic solids

2.2.2 Specifications

There are international 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, 2017) and the Food Chemicals Codex (FCC) (USPC, 2018). These specifications are included in the primary sources listed in section S3—2 of Schedule 3 of the Code and

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enzymes used as a processing aid must meet either of these specifications. Schedule 3 of the Code also includes specifications for arsenic and metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

Table 1 provides a comparison of the analysis of three batches of the carboxypeptidase enzyme with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, the enzyme meets all relevant specifications. Certificates of analysis have been provided which confirm the results below.

Table 1 Analysis of enzyme carboxypeptidase compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes (three non-sequential batches)

Analysis	Results from Applicant	JECFA	Food Chemicals Codex	Australia New Zealand Food Standards Code (section S3-4)
Lead (mg/kg)	ND	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	ND	-	-	≤1
Cadmium (mg/kg)	ND	< 0.5	-	≤1
Mercury (mg/kg)	ND	< 0.5	-	≤1
Coliforms (cfu/g)	<4	≤30	≤30	-
<i>Salmonella</i> (in 25 g)	ND	Absent	Negative	-
<i>E. coli</i> (in 25 g)	ND	Absent	-	-
Antimicrobial activity	ND	Absent	-	-

ND: Not detected; CFU: Colony-forming unit

Note: Analysis was performed on three batches of enzyme preparation.

2.3 Technological purpose of the enzyme

Carboxypeptidases belong to the hydrolase enzyme class, specifically the peptidase or protease subclass as they act on peptide bonds. They hydrolyse proteins into shorter proteins/peptides and free amino acids by preferential release of a C-terminal arginine or lysine residue (BRENDA:EC3.4.16.6, 2022). Novozymes' carboxypeptidase is a serine carboxypeptidase as it requires an essential serine to catalyse the reaction. It is a carboxypeptidase D, which means that it will preferentially react with basic groups (Remington 2013). Novozymes states the enzyme is intended to be used in the manufacture

and/or processing of proteins, yeast and flavourings; the manufacture of bakery products; and brewing.

Proteases such as carboxypeptidase can be used to convert proteins into protein hydrolysate, which has high nutritional value and can be used in various food products. The enzymes can also modify the properties of proteins including improving solubility and stability in acidic environments and at different temperatures (Sharma et al 2019). Carboxypeptidase is used to modify flavourings by reducing the bitterness of oligopeptides (Song et al 2021), and can be used to generate glutamate from proteins which adds umami to food products. In yeast processing, carboxypeptidase also hydrolyses proteins into smaller proteins and peptides for use as ingredients. It alters the elasticity of gluten and reduces the amount of water that is able to be retained. This leads to changes in dough viscosity making a softer and more machinable dough (Pourmohammadi and Abedi 2021). In brewing, carboxypeptidase is used in the breakdown of the endosperm cell wall of barley grains, making the starch and protein inside the cell accessible for further processing (Bamforth et al. 1979).

Use of commercial enzyme preparations should follow Good Manufacturing Practice (GMP), where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The conditions of the proposed use of the enzyme in food processing will depend on a number of factors including the nature of the application and the individual food manufacturers' production processes. The optimum use level should be assessed and adjusted using trials that reflect their particular processes.

The applicant provided a description of the method used for determining enzyme activity, which is CCI.

2.4 Technological justification of the enzyme

As outlined above, carboxypeptidase D is used to catalyse the breakdown of proteins, releasing shorter proteins/peptides and amino acids. The removal of certain amino acids from peptides in this fashion improves flavour by reducing bitterness. It can be used to produce and modify protein hydrolysates. In the manufacture of bakery products, carboxypeptidase improves the quality of the dough by breaking down gluten. Carboxypeptidase releases starch and protein from barley grains for brewing by breaking down the endosperm cell wall. Its use as requested by the applicant is therefore technologically justified.

2.5 Food technology conclusion

FSANZ concludes that the proposed use of carboxypeptidase from GM *A. oryzae* as a processing aid in the manufacture and/or processing of proteins, yeast and flavourings; the manufacture of bakery products; and brewing is technologically justified. This is because it is consistent with its known technological function of hydrolysis of proteins into shorter proteins/peptides, and free amino acids by preferential release of a C-terminal arginine or lysine residue.

Analysis of the evidence provides adequate assurance that the proposed use of this enzyme, at a level not higher than necessary to achieve the desired enzyme reaction under GMP usage levels, is technologically justified.

Carboxypeptidase performs its technological purpose during the production of food and is not performing a technological purpose 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 objective of this safety assessment is 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 CCI, so full details cannot be provided in this public report.

3.1 History of use

3.1.1 Host organism

The enzyme production strain is derived from a parental strain of *A. oryzae* IFO 4177 (synonym A1560) obtained from the Institute for Fermentation in Osaka, Japan. Novozymes has used this parental strain for the development of production strains for the manufacture of enzyme processing aids for many years. FSANZ has assessed the safety of this organism in previous applications including A561 (2006), A606 (2008) and A1246 (2022). No safety concerns were noted in these assessments.

The applicant provided commercial-in-confidence evidence that adequately demonstrated that the production strain was correctly identified as *A. oryzae*.

A. oryzae cannot produce aflatoxins, aspergillilic acid, and flavimine (Frisvad et al 2018). It is recognised that certain strains of *A. oryzae* are capable of producing toxic secondary metabolites including cyclopiazonic acid, Kojic acid and β -nitropropionic acid (Frisvad et al 2018). The host strain has been genetically modified to remove genes required for the production of these metabolites. Analytical analysis of the enzyme product did not detect the presence of the three metabolites or aflatoxin B1.

3.1.2 Gene donor organism

The gene for the carboxypeptidase enzyme was isolated from *A. oryzae* strain A1560 (CBS 205.89).

3.2 Characterisation of the genetic modification(s)

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

The enzyme expression cassette was constructed in an *E. coli* plasmid vector using standard molecular biology techniques. The constructed cassette that was transformed into the *A. oryzae* recipient strain consisted of the *A. oryzae* carboxypeptidase gene under the control of an *Aspergillus* sp. promoter and terminator. The final production strain was selected based on phenotypic characteristics.

3.2.2 Characterisation of inserted DNA

Details provided by Novozymes demonstrated that the expression cassette was incorporated into the chromosome of the *A. oryzae* production strain and that no antibiotic resistance genes remained.

3.2.3 Genetic stability of the inserted gene

The stability of the introduced DNA in the production strain was demonstrated by characterising three separate fermentations. The data presented indicated that the production strain is genetically stable and suitable for industrial fermentation.

3.3 Safety of carboxypeptidase

3.3.1 History of safe use of the enzyme

No history of use of this carboxypeptidase is available. The applicant describes this enzyme as a newly developed enzyme.

3.3.2 Bioinformatics concerning potential for toxicity

A recent (2020) toxin homology search was conducted, by comparing the amino acid sequence of the carboxypeptidase enzyme with the sequences of proteins annotated as toxins in the UNIPROT¹ database. The search was conducted using the sequence alignment program ClustalW 2.0.10. Homology was low, not exceeding 17.1%.

¹ [UniProt](#)

3.3.3 Toxicology data

3.3.3.1 Animal studies

*90-day oral gavage study of carboxypeptidase in rats (Covance 2020, unpublished study).
Regulatory status: GLP, conducted in compliance with OECD guideline 408*

The test article for this study was the carboxypeptidase, batch PPJ60566, which differed from the commercial preparation in that stabilizers had not been added. The test article was supplied as a frozen liquid. Dose concentration was confirmed in samples collected in weeks 1, 4, 6 and 13 of the in-life phase.

The test system comprised male and female Han Wistar rats.

During the study rats were pair-housed under standard laboratory environmental conditions and provided with feed and water *ad libitum*. Rats were assigned, 10/sex/group, to four groups. The control group was gavaged daily with reverse osmosis water at 10 mL/kg bw. The high dose group was gavaged daily with the undiluted test article at 10 mL/kg bw, representing a dose rate of 2,220 mg TOS/kg bw/day. The low and mid-dose groups were gavaged daily with 5 and 7.5 mL/kg bw of the undiluted test article respectively, resulting in dose rates of 1,110 and 1,665 mg TOS/kg bw/day.

Parameters measured during the study included survival, clinical and post-dose observations, functional observational battery, oestrous activity, body weights and bodyweight changes, food consumption, ophthalmology, clinical pathology, gross pathology, and histopathology. The functional observational battery included weekly detailed observational measurements, locomotor activity measurements in Week 11, and in Week 12, quantitative assessments of grip strength and hindlimb foot splay. Ophthalmic examinations were performed on all rats prior to study start, and on rats in the control and 2,220 mg TOS/kg bw/day groups in Week 13. Body weights were recorded prestudy and weekly during the in-life phase, while food consumption was measured twice weekly. Oestrous activity was assessed from vaginal smears collected from all females on the day of scheduled termination. Clinical pathology parameters included haematology, clinical chemistry, coagulation assessments, and thyroid hormone levels. Rats were subject to detailed necropsy with fresh organ weights recorded for adrenals, brain, gonads and accessory sex glands, heart, liver, kidneys, pituitary, spleen, thymus, thyroid, and uterus. A comprehensive list of organs and tissues were preserved for histopathology. All preserved tissues were examined from the control and high dose group, and qualitative assessments of spermatogenesis and interstitial testicular structure were assessed from the same groups. Histological examination of the low- and mid-dose groups were limited to gross lesions, and vaginas of females.

One female rat in the control group was removed from the study in moribund condition on Day 8. This rat was found on necropsy to have a perforated oesophagus and excess fluid in the thoracic cavity, consistent with gavage accident. All other rats survived to scheduled termination and no adverse effects attributable to carboxypeptidase were observed at any dose level. It was concluded that the No Observed Adverse Effect Level (NOAEL) was 2,220 mg TOS/kg bw/day, the highest dose tested.

3.3.3.2 Genotoxicity studies

Genotoxicity studies included a bacterial reverse mutation assay (Ames test) and an *in vitro* micronucleus test. Both studies were conducted using carboxypeptidase batch PPJ55674. As for the test article used for the 90-day rodent study, this test article had not had stabilisers added but was otherwise representative of the commercial preparation.

Bacterial reverse mutation assay of carboxypeptidase (Ballantyne 2019, unpublished study). Regulatory status: GLP, conducted in general compliance with OECD Guideline 471.

This study was conducted in general compliance with OECD Guideline 471, but using the “treat and plate” method, which is not described in any Guideline, rather than the plate incorporation method. The “treat and plate” method was used to avoid artefacts due to growth stimulation.

The test system comprised the *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* strain WP2 *uvrA* pKM101. The solvent and negative control article was purified water. Appropriate positive control articles, as recommended in the Guideline, were used. Three separate experiments were conducted.

The first experiment was a dose range-finding study. Treatment of all the test strains was conducted, with and without addition of S9 mix, at carboxypeptidase concentrations of 16, 50, 160, 500, 1600 and 5000 µg TOS/mL. Following these treatments, no evidence of toxicity was observed on any of the test plates.

In Experiment II, treatment of all test strains was conducted in triplicate, with and without S9 mix, at carboxypeptidase concentrations of 160, 300, 625, 1250, 2500 and 5000 µg TOS/mL. Apparent cytotoxic effects were observed at 160 and 300 µg TOS/mL in strain TA98 in the absence of S9, and at 1250 µg TOS/mL in strain TA1535 in the absence of S9, but no concentration-response relationship was evident. It was concluded that the apparent effects were due to loss of bacterial cells during washing. The loss of cells was not severe enough to adversely affect mutation assessment.

Small increases in numbers of revertant colonies of test strain TA98, only in the presence of S9, were observed at the highest test concentration in Experiment II. A third experiment was therefore conducted with the same strain, with inclusion of S9. The increases observed in Experiment II were not repeatable.

Negative control plates and appropriate positive control plates were run in triplicate, parallel to the test assays. For all test strains, 2-aminoanthracene was used as the positive control article when S9 was included. When S9 was not present, the positive control articles used for comparison were 2-nitrofluorene for TA98, 4-nitroquinoline 1-oxide for TA100, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for TA1535 and WP2 *uvrA* pKM101, and ICR-191 mutagen for TA1537. No increases in revertant colonies were observed with any negative control cultures, and the expected significant increases in revertant colonies were observed with all positive control articles, confirming the validity of the assay. It was concluded that carboxypeptidase is not mutagenic under the conditions of the assay.

In vitro human lymphocyte micronucleus assay (Whitwell 2019, unpublished study). Regulatory status: GLP; conducted in compliance with OECD Guideline 487.

The test system for this assay comprised human lymphocyte cultures from the pooled blood of two male donors. The vehicle negative control article was purified water. Tests were conducted in duplicate, and with and without S9 mix for metabolic activation. Mitomycin C and vinblastine were used as clastogenic and aneugenic positive control articles respectively in the absence of S9 mix, and cyclophosphamide was used as a clastogenic positive control article in the presence of S9 mix. Concentrations of the test article ranged from 0 to 5000 µg TOS/mL. Concentrations for the definitive assay were based on preliminary observations of the effects of the test article on the replication index, and on the presence or absence of cytotoxicity.

Short exposure (3+21 hour) treatment of cells with carboxypeptidase, with or without S9, did not result in increased frequencies of micronucleated binucleate (MNBN) cells when compared to negative controls. However, a small but statistically significant ($p \leq 0.05$) increase in MNBN cells was observed in one of two cultures following extended 24+24-hour treatment in the absence of S-9 at the intermediate concentration of 4000 µg TOS/mL. A second experiment was conducted to repeat these treatment conditions, and the increase in MNBN cells observed in the first definitive experiment did not occur. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei, confirming the validity of the assay.

It was concluded that phospholipase A1 did not induce biologically relevant increases in micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of metabolic activation.

3.3.4 Potential for allergenicity

Recent (2020) bioinformatics searches were conducted using the AllergenOnline² database. Searches included 35% identity over 80 amino acids with and without scaling, full-length alignment, and 8-amino acid search.

A match identified in both 80-amino acid searches was P08819, a serine carboxypeptidase II found in wheat (*Triticum aestivum*), with up to 50.8% identity. Using the full-length search, homology with the same allergen was 23.5%. One 100% identity with the same allergen was identified using the 8-amino acid search. P08819 is included in the database as an allergen based on *in vitro* IgE evidence.

Sequence homology of 53.6% with Api m 9.0101 was also identified in the 80-amino acid searches, although identity over the full length is only 22.9%. This is a serine carboxypeptidase produced by the honeybee, *Apis mellifera*, in the venom gland. No literature concerning this putative allergen was found, and it may be included in the database

² [AllergenOnline](#)

based on sequence homology. It is described by the WHO/IUIS Allergen Nomenclature Sub-Committee³ as being an allergen by injection.

The threshold of 35% identity over 80 or more amino acids is used for screening purposes in a weight of evidence approach that is based on scientific advice issued by the FAO/WHO in 2001 which informed the development of the Codex guidelines. Since that time further evidence indicates that the 35% threshold is overly conservative and is prone to false positive findings in relation to the potential for cross-reactivity. A conventional FASTA alignment over the entire length of the protein produces fewer false positive findings and equivalent false negative rates compared to the 80 amino acid search (Ladics et al 2007). FSANZ therefore considers the results of a full-length search to be more reliable and meaningful than the 80 amino acid search when comparing identities of proteins to allergens. When the bioinformatic comparison for the enzyme was measured over the full length of the protein, the % identity was found to be 23.5% for P08819 and 22.9% for Api m 9.0101, which are not considered to be biologically meaningful. In general, <50% amino acid identity among proteins rarely results in antigenic cross-reactivity. Greater than >70% identity is necessary before there is a high risk of cross-reactivity (Ladics et al 2014).

3.3.5 Assessments by other regulatory agencies

No assessments by other regulatory agencies are available. The applicant states that the enzyme has not been assessed by other regulatory authorities.

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 of the TOS from the carboxypeptidase 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 for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO 2021) for dietary exposure assessments for processing aids.

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

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day

³ www.allergen.org

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- 50% of solid food is processed
- on average, all processed food contains 25% proteins (or protein-derived dry matter)
- all solid foods contain the highest use level of 1,796 mg TOS/kg protein concentrate or isolate
- 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 processed non-milk beverages contain 12% protein hydrolysates
- all non-milk beverages contain the highest use level of 33.6 mg TOS/kg raw material (cereal)
- all of the TOS from the enzyme preparation remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 5.7 mg TOS/kg body weight/day.

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

- The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst-case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations for solid food and non-milk beverages is 11.3 mg TOS/kg body weight/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes the assumption that all the TOS from the enzyme preparation remains in the final foods and beverages, whereas the applicant has stated the TOS is likely to be diluted or removed during processing and 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 in the assessment of carboxypeptidase from *GM A. oryzae* under the proposed use conditions. *A. oryzae* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. The *A. oryzae* host is neither pathogenic or toxigenic. Analysis of the modified production strain confirmed the presence and stability of the inserted DNA.

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The enzyme did not show significant homology with known toxins, or with known food allergens. Results of genotoxicity assays were negative. A NOAEL of 2,220 mg TOS/kg bw/day was identified in a 90-day oral toxicity study in rats. The TMDI was calculated by FSANZ to be 11.3 mg TOS/kg bw. A comparison of the NOAEL and the TMDI results in a large Margin of Exposure (MOE) of approximately 200. 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

Aunstrup K (1979) Production, Isolation, and Economics of Extracellular Enzymes in Applied Biochemistry and Bioengineering, Volume 2, Enzyme Technology, Eds. Wingard, L.B., Katchalski-Katzir, E. and Goldstein, L, pp. 28–68

Bamforth CW, Martin HL, Wainwright T (1979) A role for carboxypeptidase in the solubilization of barley β -glucan. *Journal of the Institute of Brewing*. Vol.85, pp. 334–338

BRENDA:EC3.4.16.6 <https://www.brenda-enzymes.org/enzyme.php?ecno=3.4.16.6>
Accessed on 26 September 2022

Douglass JS, Barraj LM, Tennant DR, Long WR, Chaisson CF (1997) Evaluation of the Budget Method for screening food additive intakes. *Food Additives and Contamination* 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 LLH, 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*. *Appl Microbiol Biotechnol* 102:9481–9515.

IUBMB (2018) EC 3.4.16.6 <https://iubmb.qmul.ac.uk/enzyme/EC3/4/16/6.html> Accessed 26 September 2022

JECFA (2017) Combined compendium of food additive specifications (FAO JECFA Monograph 1) <http://www.fao.org/docrep/009/a0691e/A0691E03.htm>

Ladics GS, 1, Bannon GA, Silvanovich A and Cressman RF (2007). Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search

for the elucidation of potential identities to known allergens. *Molecular Nutrition & Food Research* 51, 985 - 998

Ladics GS, Fry J, Goodman R, Herouet-Guicheney C, Hoffmann-Sommergruber K, Madsen CB, Penninks A, Pomés A, Roggen EL, Smit J and Wal J-M (2014) Allergic sensitization: screening methods. *Clinical and Translational Allergy* 4:13

Pourmohammadi K and Abedi E (2021) Hydrolytic enzymes and their directly and indirectly effects on gluten and dough properties: An extensive review. *Food Science and Nutrition* 9:3988-4006

Remington SJ (2013) *Handbook of Proteolytic Enzymes (Third Edition)*. Chapter 755 – Serine Carboxypeptidase D. p 3418–3421

Sharma M, Gat Y, Arya S, Kumar V, Panghal A, and Kumar A (2019) A Review on Microbial Alkaline Protease: An Essential Tool for Various Industrial Approaches. *Industrial Biotechnology*. 15(2): 69–78. <http://doi.org/10.1089/ind.2018.0032>

Song P, Xu W, Zang Y, Wang F, Zhou X, Shi H and Feng W (2021) A new carboxypeptidase from *Aspergillus niger* with good thermostability, pH stability and broad substrate specificity. *Scientific Reports* 11:18745