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

25 October 2019
[100-19]

Supporting document 1

Risk and technical assessment report – Application A1169

Alpha-Glucosidase from *Trichoderma reesei* as a processing aid (Enzyme).

Executive summary

DuPont Australia Pty Ltd submitted an application to Food Standards Australia New Zealand (FSANZ) seeking to permit alpha-glucosidase (α-glucosidase) from *Trichoderma reesei,* which is genetically modified to express the α-glucosidase gene from *Aspergillus niger,* as a processing aid.

The proposed use of the α-glucosidase is for the production of biochemicals such as monosodium glutamate (MSG) and other amino acids, organic acids (e.g. lactic acid, citric acid and succinic acid), potable alcohol, isomalto-oligosaccharides (IMO) and other sweeteners and lysine. These are not foods for sale, themselves but are used as ingredients in foods for sale.

The food technology assessment concluded that α-glucosidase, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. α-Glucosidase performs its technological purpose during the processing and production of foods and is therefore appropriately categorised as a processing aid. The enzyme preparation meets international purity specifications.

T. reesei has a long history of safe use as the source of enzyme processing aids, including several that are already permitted in the Code. This fungus is not toxigenic or pathogenic. No extraneous coding genetic material is carried across from the donor organism or through the large number of steps leading to the final genetic modification. The modification involving the insertion of the α-glucosidase gene has been shown to be stably inherited.

The enzyme α-glucosidase from genetically modified *T. reesei* shows no significant homology with any known allergens, venoms or toxins.

No evidence of genotoxicity was found in a bacterial reverse mutation assay or in a chromosomal aberration assay in human lymphocytes. In an 18-week study in rats, the No Observed Effect Level (NOEL) was 63.64 mg/kg bw/day α-glucosidase (expressed as total protein), the highest dose tested. This dose corresponds to 77.2 mg TOS/kg bw/day. The Theoretical Maximal Daily Intake (TMDI) in consumers under the proposed conditions of use is 0.443 mg/kg bw/day TOS. Consequently, the Margin of Safety (MoS) between the TMDI and the NOEL in rats is 174.

In the absence of any identifiable hazard an Acceptable Daily Intake ‘not specified’ is appropriate, and therefore a dietary exposure assessment is not required.

Nutrient raw materials used in the bacterial fermentation process to produce α-glucosidase include soy protein and glucose derived from wheat. Therefore the enzyme preparation may contain traces of wheat or soy. DuPont has estimated that the highest amount of soy protein or wheat protein in the final food would be 2-3 ppb and 5 ppb, respectively.

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1 Introduction

DuPont Australia Pty Ltd has made an application to FSANZ seeking permission for a new microbial source for the already permitted enzyme, α-glucosidase (EC 3 2 1.20), as a processing aid.

α-Glucosidase is produced from fermentation of a genetically modified (GM) strain of *Trichoderma reesei* which expresses the α-glucosidase gene from *Aspergillus niger.* If approved, α-glucosidase will be used in the production of of biochemicals such as monosodium glutamate (MSG) and other amino acids, organic acids (e.g. lactic acid, citric acid and succinic acid), potable alcohol, isomalto-oligosaccharides (IMO) and other sweeteners and lysine. These are not foods for sale, themselves but are used as ingredients in foods for sale.

α-Glucosidase will be used as a processing aid at a level consistent with Good Manufacturing Practice (GMP) and provides no technical function in the final food. It has been determined GRAS by a panel of scientific experts in the United States.

There are two permissions for α-glucosidase as a processing aid in the Australia New Zealand Food Standards Code (the Code), however there is no permission for α-glucosidase sourced from a GM strain of *T. reesei.* Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market safety assessment.

1.1 Objectives of the assessment

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

- determine whether the proposed purpose is clearly stated and that α-glucosidase achieves its technological function in the quantity and form proposed to be used as a food processing aid

- evaluate potential public health and safety risks that may arise from the use of α-glucosidase in the manufacture of potable alcohol, lysine, organic acids, MSG and other biochemicals, production of IMO and other sweeteners.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

Information regarding the identity of the enzyme provided in the application has been verified using the appropriate internationally accepted reference for enzyme nomenclature, the International Union of Biology and Molecular Biology (IUBMB 2018).

Accepted name:	α-glucosidase
IUBMB[1]/EC[2] number:	3.2.1.20
CAS registry number[3]:	9001-42-7
Common names:	Acid maltase; Glucoinvertase; Glucosidosucrase; Lysosomal α-glucosidase; Maltase; Maltase-glucoamylase maltase; α-Glucopyranosidase; Glucosidoinvertase; α-D-glucosidase; α-glucoside hydrolase; α-1,4-glucosidase
Reaction:	Hydrolysis of terminal, non-reducing (1->4)-linked alpha-D-glucose residues with release of alpha-D-glucose

α-Glucosidase was also identified historically as Transglucosidase (EC 2.4.1.24), with synonyms oligoglucan-branching glycosyltransferase; 1, 4-alpha-D-glucan 6- alpha-D-glucosyltransferase; T-enzyme; D-glucosyltransferase; and 1, 4-alpha-D-glucan: 1, 4- alpha-D-glucan (D-glucose) 6-alpha-D-glucosyltransferase.

2.1.2 Technological purpose of the enzyme

α-Glucosidase catalyses the hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of α-D-glucose (IUBMB 2018). This reaction catalyses the conversion of non-fermentable sugars in molasses such as raffinose and stachyose to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol. The same reaction is also used in yeast fermentation to manufacture potable alcohol, organic acids (e.g. lactic acid, citric acid), MSG and other biochemicals.

α-Glucosidase also catalyses a synthetic or transfer reaction, which transfers a glucosyl residue from the substrate (maltose) to form oligosaccharides having alpha-D 1, 6 linkages like isomaltose, panose, isomaltotriose and higher branched oligosaccarides together with varying amounts of glucose. The main intention of the synthetic or transfer reaction of α-glucosidase is to facilitate the production of IMO syrups “in-situ” or from starch.

α-Glucosidase performs its technological purpose during production and manufacture of food ingredients, after which it is inactivated and therefore not performing a technological function in the final food.

2.1.3 Technological justification of the enzyme

There are currently permissions for α-glucosidase (EC 3.2.1.20) from *A. oryzae* and *A. niger*

[1] International Union of Biochemistry and Molecular Biology.
[2] Enzyme Commission, internationally recognised number that provides a unique identifier for enzymes
[3] Chemical Abstracts Service Registry Number, internationally recognised number that provides a unique identifier for organic and inorganic chemical substances

within the table to subsection S18—4(5), to be used in the manufacture of all foods. However, α-glucosidase from this particular microbial source, the subject of this application, is not currently permitted.

Two reactions are catalysed by α-glucosidase; hydrolysis and transfer. The hydrolysis reaction catalyses the conversion of non-fermentable sugars in molasses such as raffinose and stachyose to sucrose, galactose, glucose and fructose, which can then be fermented into alcohol. The same reaction is also used in yeast fermentation to manufacture potable alcohol and organic acids (e.g. lactic acid, citric acid and succinic acid) and MSG.

The transfer reaction transfers a glucosyl residue from the substrate (maltose) and forms oligosaccharides having alpha-D 1,6 linkages like isomaltose, panose, isomaltotriose and higher branched oligosaccarides together with varying amounts of glucose. The main intention of the synthetic or transfer reaction of α-glucosidase is to facilitate the production of IMO syrups *in-situ*, or from starch. Its use has also some beneficial effects on the final syrup, such as:

- providing a mild sweetness (about half as sweet as sucrose)
- providing a lower viscosity than maltose syrups
- preventing staling (retrogradation) of starchy foods and retaining a suitable moisture level in foods, which helps to control microbial growth.

Historically, the enzyme is also described as transglucosidase in the synthetic or transfer reaction, where it was also utilised a processing aid, therefore not present or active in the final food or present in negligible amounts with no technical function in the final food.

The commercial enzyme preparation is a brown liquid, with examples of marketing names including FERMENZYME® TL and FERMENZYME® TL FG.

2.1.4 Substrate specificity

In the hydrolysis reaction, α-glucosidase hydrolysis of terminal, non-reducing (1->4)-linked alpha-D-glucose residues with release of alpha-D-glucose (Figure 1).

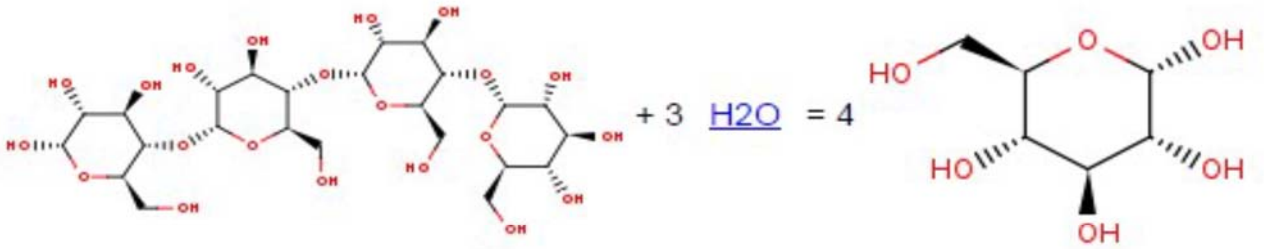

Figure 1 *α-glucosidase hydrolysis reaction*

In the synthetic or transfer reaction, α-glucosidase hydrolyses and transfers an alpha-D-glucosyl units of oligosaccharides and convert 1, 4 glucosidic linkage to 1, 6 glucosidic linkages. Transfer occurs most frequently to HO-6 (the hydroxy group at the 6-position), producing isomaltose from D-glucose, and panose from maltose (Figure2).

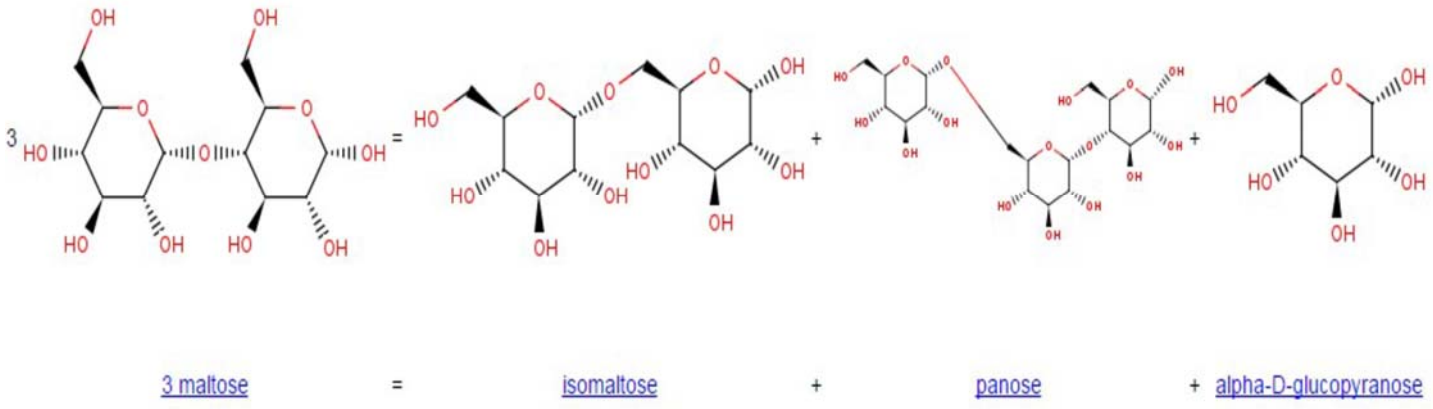

Figure 2 *α-glucosidase transfer reaction*

The transfer activity is favoured under the conditions of high concentration of mono- and oligosaccharides and low water activity.

2.1.5 Activity and stability

In the hydrolysis reaction, the activity of the α-glucosidase is defined in U (units)/g. This activity is measured based on the ability of α-glucosidase enzyme to catalyse the hydrolysis of *p*-nitrophenyl-alpha-D-glucopyranoside (PNPG) to glucose and *p*-nitrophenol. At an alkaline pH, the nitrophenol forms a yellow colour that is proportional to α-glucosidase activity and is monitored at 420nm via the use of an enzyme standard.

In the transfer reaction, the activity of the α-glucosidase is defined in TGU (Transglucosidase Unit). TGU is defined as the amount of enzyme which will produce one micromole of trisaccharide per minute under assay conditions.

DuPont provides information in the application showing that α-glucosidase exhibits optimum activity around 58-70°C, with activity observed from 30°C until 90°C. It is thermally stable for 30 minutes at 60°C, while it is inactivated after 30 minutes of incubation at 71°C. The optimum pH for α-glucosidase activity is below pH 5.6, although the enzyme exhibits activity at pH <8.3. More detail on the assays to determine the optimum temperature and pH conditions can be found on pp4-7 of Appendix A of the application.

DuPont's α-glucosidase enzyme preparation was shown to be stable during storage for 24 months with close to 90% activity remaining.

2.1.6 Usage levels

Uses of commercial enzyme preparations are typically in accordance with GMP, whereby use is at a level that is not higher than the level necessary to achieve the desired enzymatic reaction. As such food manufacturers adjust the usage levels depending on the food use, the type and quality of the raw materials used and the enzyme supplier's recommendations (Table 2).

Table 2 *Recommended use levels for α-glucosidase*

Reaction type	Application	Recommended use levels (% w/w)
Hydrolysis	Organic acids such as lactic acid, citric acid and succinic. MSG	2-4
	Potable alcohol.	.0006-.002
Transfer	IMO & other sweeteners	0.50-1.50

2.2 Manufacturing process

2.2.1 Production of the enzyme

α-Glucosidase is produced by a submerged fermentation of *T. reesei* to express the α-glucosidase gene from *A. niger* using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation and 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 (Figure 3).

Whilst full details of the raw materials used for the production were provided by DuPont, this information is proprietary and as such, “Confidential Commercial Information” was granted by FSANZ.

The production of DuPont’s α -glucosidase 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.

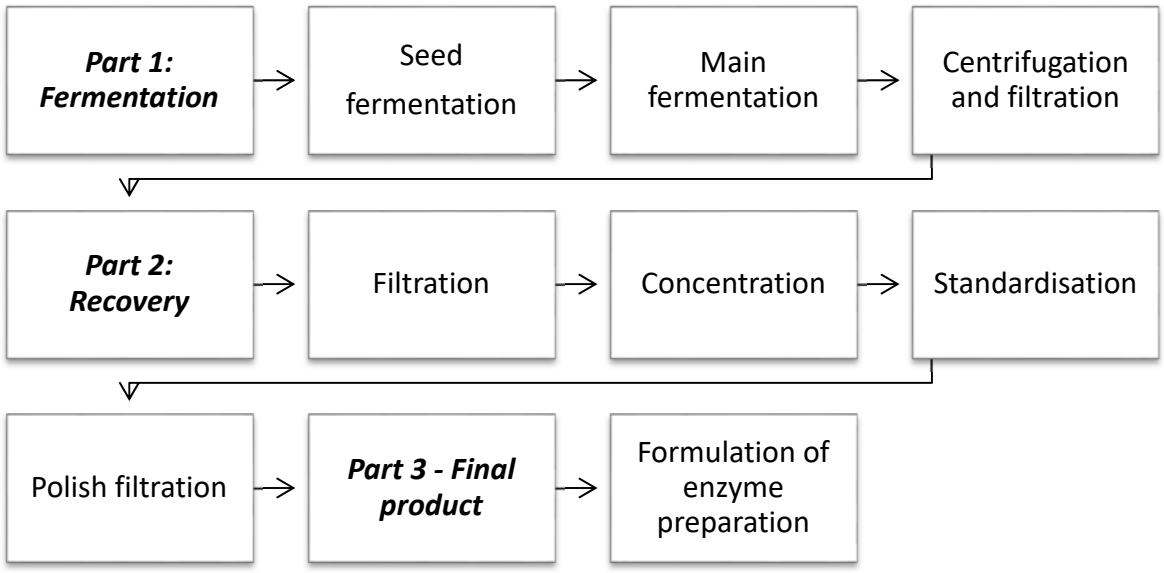

Figure 3 *Manufacturing process for DuPont's α-glucosidase*

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) Compendium of Food Additive Specifications (JECFA 2016) and the United States Pharmacopeial Convention (USPC) Food Chemicals Codex 11th edition (USPC 2018). Both of these specification sources are primary sources listed in section S3—2 of the Code. Enzyme preparations must meet these specifications.

Table 3 provides a comparison of three representative batch analysis of α-glucosidase with the international specifications established by JECFA and USPC, as well as those detailed in the Code (as applicable).

Table 3 *Product specifications for commercial α-glucosidase enzyme preparation*

Analysis	Enzyme batch analysis			Specifications		
				JECFA	USPC	the Code
Lead (mg/kg)	< 5.0	< 5.0	< 5.0	≤5.0	≤5.0	≤2.0
Arsenic (mg/kg)	<3.0	<.3.0	< 3.0	-	-	≤1.0
Cadmium (mg/kg)	< 0.5	< 0.5	< 0.5	-	-	≤1.0
Mercury (mg/kg)	< 0.5	< 0.5	< 0.5	-	-	≤1.0
Total coliforms (cfu/g)	< 10	<1.0	<1.0	≤30	≤30	-
Salmonella (in 25 g)	Negative	Negative	Negative	Absent	Absent	-
Enteropathic *E. coli* (in 25 g)	Negative	Negative	Negative	Absent	-	-
Antibiotic activity	Negative	Negative	Negative	Absent	-	-

[a] ND = Not detected

[b] DL = Detection limit

Based on the above results for the enzyme preparation, where there is no specification under section S3—2 or S3—3, or if the monographs referred to in those sections do not contain a specification for identity and purity of a substance relating to arsenic or heavy metals, the specification must meet the conditions in S3—4. DuPont should note that any α-glucosidase enzyme preparation being marketed in Australia and New Zealand must meet these conditions.

2.3 Food technology conclusion

FSANZ concludes that the stated purpose of this enzyme preparation, namely for use as a processing aid in the production of potable alcohol, lysine, organic acids, MSG and other biochemicals, production of IMO and other sweeteners is clearly articulated in the application. The evidence presented to support the proposed uses provides adequate assurance that the α-glucosidase enzyme preparation, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. α-Glucosidase performs its technological purpose during production and manufacture of foods ingredients, after which it is inactivated thereby not performing a technological function in the final food. It is therefore appropriately categorised as a processing aid. The α-glucosidase enzyme preparation needs to meet international purity specifications, or those set out in the Code to be sold in Australia and New Zealand.

3 Safety assessment

3.1 Objective for safety assessment

The objective of this safety assessment for α-glucosidase from *T. reesei* which is genetically modified to express the α-glucosidase gene from *A. niger,* is to evaluate any potential public health and safety concerns that may arise from the use of this enzyme as a processing aid. Consideration is given to the history of use of the host and gene donor organisms, characterisation of the genetic modification(s), and safety of the enzyme.

3.2 History of use

3.2.1 Host organism

T. reesei is a common 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). In humans *T. reesei* is not pathogenic. Although some *T. reesei* strains can produce mycotoxins, most industrial production strains do not produce mycotoxin or antibiotics under conditions used for enzyme production (Nevalainen *et al.,* 1994; Blumenthal 2004).

FSANZ has previously assessed the safety of *T. reesei* as the source organism for a number of enzymes used as processing aids. Schedule 18 lists the following permitted enzymes derived from *T. reesei:* cellulase, endo-1,4-beta-xylanase, β-glucanase, hemicellulase multicomponent enzyme and polygalacturonase or pectinase multicomponent enzyme.

3.2.2 Gene donor organism(s)

The α-glucosidase gene was sourced from *A. niger*, a filamentous fungus ubiquitous in the environment. FSANZ has previously assessed the safety of *A. niger* as a gene donor for glucose oxidase but also as the source organism for 28 endogenous enzyme products, including α-glucosidase (the enzyme being assessed in this application).

3.3 Characterisation of the genetic modification(s)

Full details of the genetic modification to the production organism were provided to FSANZ for assessment but cannot be disclosed as they are confidential commercial information. A summary of FSANZ's safety assessment of that information is given below.

3.3.1 Description of DNA to be introduced and method of transformation

A single expression cassette was generated and host transformation performed using standard methodologies. The expression cassette contained two coding regions. The first coding region contains the α-glucosidase gene, flanked by well characterised promoter and terminator sequences. The second coding region contains a selective marker gene commonly used in yeast.

3.3.2 Characterisation of inserted DNA

Southern blot analyses were used to confirm the presence of the inserted DNA in the hosts' genome and to determine the gene copy number. Indirectly, the analyses showed there was no rearrangement of the inserted DNA.

3.3.3 Genetic stability of the inserted gene

The stability of the inserted gene was demonstrated by comparing samples taken before and after prolonged fermentation under conditions similar to commercial production. Southern blot analyses showed the inserted DNA was maintained across the generations and did not undergo rearrangement.

3.4 Safety of alpha-glucosidase

3.4.1 History of safe use of the enzyme

This enzyme has been used in the USA for production of IMO since 2009, and for production of organic acids and potable alcohol since 2014. No adverse effects have been reported in association with these uses.

3.4.2 Bioinformatics concerning potential for toxicity

Bioinformatic analyses for toxin homology of the amino acid sequence of the mature enzyme were conducted. A BLAST search against the Uniprot annotated Protein Knowledge database with a threshold E-value of 0.1 yielded numerous hits of α-glucosidases, but none of the top 1000 hits was annotated as a toxin or a venom. A BLAST search was also conducted against the Uniprot animal toxin database. This yielded no matches.

3.4.3 Toxicology studies

Two study reports conducted with the α-glucosidase that is the subject of the application were submitted. No test article-related adverse effects were observed in either study.

Acute toxicity study in Wistar rats (Harlan Laboratories, Study No. 57481, 2009). Regulatory status: GLP; compliant with OECD Guideline No 423 (2001) and Commission Regulation (EC) 440 (2008)

The test subjects for this study were female Wistar rats, aged between 8 and 10 weeks at time of treatment, and ranging in bodyweight from 167 to 181 g. Rats were group-housed, three/cage, in polycarbonate cages under standard laboratory environmental conditions. Standard rat chow was provided *ad libitum*, except during overnight fasting prior to dosing, and for 3 to 4 hours after dosing. Tap water was provided *at libitum*. Rats were randomly assigned to one of two groups, 3/group. The α-glucosidase enzyme was formulated at a concentration of 0.1 g/mL in an 0.9% saline vehicle, and administered once by oral gavage at a dose volume of 20 mL/kg bw, resulting in a dosage of 2000 mg/kg bw. Clinical observations were recorded at 0.5, 1, 2, 3, and 5 hours after dosing on Day 1, and once daily on Days 2 to 15. Morbidity/mortality checks were made twice daily on Days 2 through to 15. Individual bodyweights were recorded on Day 1 prior to dose administration, and on Days 8 and 15. Rats were killed on Day 15 using intraperitoneal injection of barbiturate, and gross necropsy was performed on each rat.

All rats survived to scheduled kill, and no abnormal clinical signs were observed. Body weights and bodyweight gains were within normal range for rats of this strain and sex. There were no abnormal findings on gross necropsy. It was concluded that the acute oral median lethal dose (LD50) for the enzyme in the rat is greater than 2000 mg/kg bw.

18-week repeat-dose oral gavage study in Wistar rats (Harlan Laboratories, Study No. 57558, 2010). Regulatory status: GLP; compliant with requirements of OECD Guideline No. 408 (1996) and Directive 96/54/EC.B.26 (1998).

Male and female Wistar rats were obtained at approximately 7 weeks and acclimatized under study conditions for five days prior to study start. Rats were housed in groups of five in polycarbonate cages under standard laboratory environmental conditions. Standard rat chow and tap water were provided *at libitum*. The test article had a stated purity of 106.4 mg/mL total protein. The control article and vehicle was 0.9% saline. Dose formulations were prepared weekly, stored refrigerated and stirred with a magnetic stirrer during administration periods. Dose formulations were analysed for concentration and homogeneity on Day 1, and for concentration in weeks 1, 6, 11, 14 and 18.

Rats were assigned to groups, 10/sex/group, and dosed once daily by oral gavage with 0, 10.64, 31.92 or 63.64 mg/kg bw/day enzyme expressed as total protein. Rats were observed for mortality/moribundity twice daily, and for cageside clinical observations once daily during acclimatization, twice daily on Days 1, 2, 3, 36, 37, 38, and once daily on all other days during the in-life phase. Detailed clinical and behavioural observations, including behaviour in a standard arena, were recorded once during acclimatization and once weekly during the in-life phase, and food consumption was recorded according to the same schedule. Grip strength and locomotor activity were measured quantitatively during Week 18. Individual body weights were recorded weekly during acclimatization and the treatment period, and immediately before the rats were killed for scheduled necropsy. Ophthalmoscopic examinations were performed during Week 18 on rats in the control and 63.64 mg/kg bw/day (high dose) groups. Rats were fasted overnight in metabolism cages, with access to water, at the end of 5 weeks and 18 weeks. Urine was collected during fasting and blood was collected from each rat at the end of the fasting period for routine hematology, clinical biochemistry and determination of coagulation and methaemoglobin. Urine was analysed for routine physical and chemical parameters. At the end of the in-life phase, rats were killed by exsanguination under anaesthesia, and gross necropsies were performed. Fresh organ weights were recorded for adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, seminal vesicles and prostate gland, spleen, testes, thymus and uterus. The list of organs preserved, processed, and stained with HE was comprehensive. Slides of organs and tissues collected from rats in the control and high dose groups were examined by the study pathologist.

There were no test article-related deaths during the study. In the 31.92 mg/kg bw/day group, one male was found dead and one female was euthanized due to dosing errors, and one control female was euthanized due to development of a mass. There were no test article-related effects on cageside or detailed clinical observations, or on grip strength or locomotor activity. The few clinical signs observed were incidental, superficial lesions and showed no dose-response relationship. Group mean daily food consumption values for treated rats were not significantly different to that of sex-matched controls. Male rats in the 63.64 mg/kg bw/day group had a slightly lower group mean bodyweight than that of control males through much of the study but this difference only reached statistical significance on one day of the study and group mean body weight gains were comparable to those of male controls. Furthermore there was no corresponding effect in females. On the contrary, the group mean bodyweight values for all the treated females significantly exceeded those of controls on some days of the study. It was therefore concluded that the lower group mean bodyweight observed in males in the 63.64 mg/kg bw/day group was not related to the treatment. There were no treatment-related differences in ophthalmoscopic findings between 63.64 mg/kg bw/day rats and control rats.

Minor variations in group mean values for haematology parameters were considered to be unrelated to treatment and generally showed no dose-response relationship. A right shift in reticulocyte maturity indices in 63.64 mg/kg bw/day males, was considered to be unrelated to treatment because related parameters were unchanged. No adverse effects of treatment were observed in group mean values for clinical biochemistry parameters or urinalysis parameters. Treatment had no effects on absolute or relative organ weights. Findings on

gross necropsy were limited to incidental lesions that showed no dose-response relationship, and no treatment-related microscopic changes were observed.

It was concluded from this study that the highest dose administered, 63.64 mg/kg bw/day enzyme, expressed as total protein, was the NOEL. This dose corresponds to 77.2 mg TOS/kg bw/day.

3.4.4 Genotoxicity assays

Two genotoxicity assays were submitted, a bacterial reverse mutation assay (Ames test) and an *in vitro* chromosomal aberration test conducted using human lymphocytes. Both genotoxicity studies were conducted using the enzyme that is the subject of this application as the test article. No evidence of genotoxicity was observed in either assay.

Bacterial reverse mutation assay (Harlan Laboratories, Study No. C57525, 2009). Regulatory status: GLP; compliant with requirements of OECD Guideline No. 471 (1997), Commission Regulation (EC) No. 440/2008 B13/14 (2008) and EPA Health Effects Test Guidelines OPPTS 870.5100 (1998).

Test systems for this study were *Salmonella typhimurium* strains TA1537, TA 98, TA 1535 and TA 100, and *Escherichia coli* strain WP2 uvrA. The solvent and negative control article for this study was deionised water. For assays conducted without metabolic activation, positive control articles were sodium azide for TA 1535 and TA 100; 4-nitro-o-phenylene-diamine for TA1537 and TA 98; and methyl methane sulfonate for WP2 uvrA. For assays conducted with the addition of S9 mix for the purpose of metabolic activation, the positive control article for use with all bacterial strains was 2-aminoanthracene.

The assay was performed in two independent experiments, both with and without S9 mix for metabolic activation. All assays were conducted in triplicate, including negative and positive control assays. Both experiments were conducted using the plate incorporation method. Concentrations of test article used in the first experiment were 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate, whereas for the second experiment the concentrations of test article were 33, 100, 333, 1000, 2500 and 5000 µg/plate. Test solution (100 µL), 500 µL S9 mix or substitution buffer, and 100 µL bacterial solution were mixed in a test tube and shaken at 37°C for 60 minutes. Overlay agar, 2.0 mL, was then added to each tube and the mixture was poured on selective agar plates. After the agar set, plates were inverted and incubated for at least 48 h at 37°C in the dark. Bacterial colonies were counted. The test article showed no evidence of precipitation or toxicity, and there was no significant increase in revertant colonies, with or without S9 mix. A significant increase in revertant colonies was observed in the presence of positive control articles, confirming the validity of the assays. It was concluded that the test article did not cause base pair changes or frameshift mutations in the experimental strains used.

In vitro *chromosomal aberration test conducted using human lymphocytes (Harlan Laboratories, Study No. C57547, 2009). Regulatory status: GLP; compliant with requirements of OECD Guideline No. 473 (1998) and Commission Regulation (EC) No. 440/2008 B10 (2008).*

Human lymphocytes were harvested from the blood of healthy female volunteers. The solvent control was deionised water. The positive control articles were ethylmethane sulfonate for assays without addition of S9 mix for metabolic activation, and cyclophosphamide for assays with addition of S9 mix. All cell cultures were set up in duplicate.

A preliminary cytotoxicity range-finder (Experiment I) was performed with 10 concentrations

of the test article, and negative and positive controls. The concentrations of test article were 32.5, 56.8, 99.5, 174.1, 304.6, 533.1, 932.9, 1632.7, 2857.1 and 5000 µg/mL, and the assays were carried out with and without S9 mix. Incubation conditions were 37°C in a humidified atmosphere with 5.5% CO_2. Exposure time to the test article was 4 hours, after which cells were washed, re-suspended in culture medium and cultured for a further 18 hours until chromosomes were prepared for examination. Colcemid was added three hours prior to chromosome preparation. There was no evidence of precipitation or cytotoxicity at any concentration of test article. Assays using the top three concentrations of test article, as well as negative and positive control assays, were examined by scoring at least 100 metaphase plates per culture structural chromosomal aberrations, and counting at least 1000 cells per culture for determination of mitotic index. No clastogenicity or increase in polyploidy metaphases was observed. A single slight increase in cells containing aberrant chromosomes, relative to historical control data, was observed in the presence of S9 mix, but there was no dose-dependence and the difference was not statistically significant. Statistically significant increases in cells with structural chromosome aberrations were observed in positive control assays.

The definitive experiment was conducted with test article concentrations ≥174.1 µg/mL without S9 mix, and ≥533.1 µg/mL with S9 mix. Without S9 mix, exposure to the test article was for the full 22 hours, whereas with S9 mix, cells were washed and resuspended after 4 hours of exposure. Incubation conditions, colcemid addition and examination of cells and chromosomes were as for Experiment I. In the presence of S9 mix, one statistically significant increase in chromosomal aberrations was observed in a culture exposed to 2857.1 µg/mL, but the value was within the range of historical controls. Statistically significant increases in cells with structural chromosome aberrations were observed in positive control assays.

It was concluded that the test article did not induce structural chromosomal aberrations in human lymphocytes *in vitro*.

3.4.5 Potential for allergenicity

A full length sequence alignment search was conducted using the Food Allergy Research and Resource Program (FARRP) AllergenOnline database, using an E-value < 0.1 as the cut-off. The same database was used to conduct an 80 amino acid sliding window search with a cut-off of ≥35% identity to known allergens. No matches were found in either search.

3.4.6 Approvals by other regulatory agencies

The US FDA responded with a No Questions letter to a GRAS notification for this α-glucosidase, under the name transglucosidase, in 2010, and under its current name in 2017.

4 Discussion

T. reesei has a long history of safe use in the source of enzyme processing aids, including several that are already permitted in the Code. This fungus is not toxigenic or pathogenic. No extraneous coding genetic material is carried across from the donor organism or through the large number of steps leading to the final genetic modification. The modification involving the insertion of the α-glucosidase gene has been shown to be stably inherited.

The enzyme α-glucosidase from genetically modified *T. reesei* shows no significant homology with any known allergens, venoms or toxins.

No evidence of genotoxicity was found in a bacterial reverse mutation assay or in a chromosomal aberration assay in human lymphocytes. In an 18-week study in rats, the No

Observed Effect Level (NOEL) was 63.64 mg/kg bw/day α-glucosidase (expressed as total protein), the highest dose tested. This dose corresponds to 77.2 mg TOS/kg bw/day. The Theoretical Maximal Daily Intake (TMDI) in consumers under the proposed conditions of use is 0.443 mg/kg bw/day TOS. Consequently, the Margin of Safety (MoS) between the TMDI and the NOEL in rats is 174.

Nutrient raw materials used in the bacterial fermentation process to produce α-glucosidase include soy protein and glucose derived from wheat. Therefore the enzyme preparation may contain traces of wheat or soy. DuPont has estimated that the highest amount of soy protein or wheat protein in the final food would be 2-3 ppb and 5 ppb, respectively.

5 Conclusions

In the absence of any identifiable hazard an Acceptable Daily Intake ‘not specified’ is appropriate, and therefore a dietary exposure assessment is not required.

6 References

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