5 February 2019
[72-19]

Supporting document 1

Technical and safety assessment report – Application A1159

Triacylglycerol lipase 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 the use of the enzyme triacylglycerol lipase (EC 3.1.1.3) as a processing aid. The enzyme is derived from a genetically modified strain of *Trichoderma reesei*, expressing the lipase 3 gene from *Aspergillus tubingensis*. The triacylglycerol lipase is intended for use in the baking and brewing industries.

The food technological assessment concluded that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The enzyme performs its technological purpose during production and manufacture of foods and is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

No extraneous coding genetic material is carried across from the donor organism or through the steps leading to the final genetic modification. The modification involving the insertion of the lipase gene has been shown to be stably inherited.

The enzyme showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay in human lymphocytes. In a 90-day oral gavage study in rats, the no observed adverse effect level (NOAEL) was the highest dose tested, 160.6 mg/kg bw/day total protein, which is equivalent to 123.15 mg/kg bw/day enzyme total organic solids (TOS). The applicant's estimated theoretical maximum daily intake (TMDI) based on the proposed uses is 0.410 mg/kg bw/day TOS. From these values, the Margin of Exposure is approximately 300.

Bioinformatic data indicate a lack of homology with known toxins or allergens, and the enzyme is unlikely to pose an allergenicity concern. The applicant has indicated that enzyme products used for bakery production may contain wheat as a carrier. The enzyme product may also contain traces of soy and gluten-containing cereals since such products can be used in fermentation media to produce the enzyme.

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. A dietary exposure assessment

was therefore not required.

FSANZ concluded there are no public health or safety concerns for the general population associated with the use of triacylglycerol lipase from *T. reesei* as a food processing aid.

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1   Introduction

Triacylglycerol lipase (EC 3.1.1.3) is an enzyme which catalyses the hydrolysis of ester bonds primarily in positions 1 and 3 of fatty acids in triglycerides with the subsequent release of fatty acids and glycerol. The enzyme is produced through a microbial fermentation, using a genetically modified (GM) strain of *Trichoderma reesei*. Triacylglycerol lipase is intended for use in the baking and brewing industries. In the baking industries the enzyme performs its technological purpose during dough and batter handling to improve dough stability and dough handling properties. In the brewing industry the enzyme performs its technological purpose during the mashing and fermentation steps by removing triglycerides (lipids) which otherwise cause problems in mash separation and yeast fermentation.

1.1   Objectives of the assessment

The objectives of this technical and safety assessment for triacylglycerol lipase were to:

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

*   evaluate any potential public health and safety issues that may arise from the use of this enzyme, produced by a genetically modified organism as a processing aid. Specifically by considering the:
    −   history of use of the host and gene donor organisms
    −   characterisation of the genetic modification(s), and
    −   safety of the enzyme.

2   Food technology assessment

2.1   Characterisation of the enzyme

2.1.1 Identity of the enzyme

The source microorganism of the enzyme is *Trichoderma reesei*. More information on the source microorganism is provided in section 3.

Information concerning the identity of the enzyme from the application has been verified using an appropriate enzyme nomenclature reference (IUBMB 2018).

Systematic name:	Triacylglycerol acylhydrolase
Accepted IUBMB[1] name:	Triacylglycerol lipase
Common names:	Lipase; triglyceride lipase; tributyrase; butyrinase; glycerol ester hydrolase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno N-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride

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[1] International Union of Biochemistry and Molecular Biology.

IUBMB enzyme nomenclature: EC 3.1.1.3

CAS[2] registry number: 9001-62-1

Reaction: Triacylglycerol + H_2O = diacylglycerol + carboxylate

The enzyme preparation can be prepared either as an amber to brown liquid, or if used for baking, as an off-white powder. The carrier for the liquid preparation is glycerol and water. The powder is sprayed dried on either potato or wheat starch. Baked products usually use wheat or other cereals containing gluten as ingredients so the requirements for potential allergen declarations are already covered by the use of ingredients. Food manufacturers need to be aware of the source of the carrier for the enzyme preparation and the potential allergen declarations.

The following information is provided in the application. The optimum temperature for enzyme activity is 30°C, with relatively high activity up to 40°C. The activity is reduced below 50% at temperatures above 45°C. The enzyme is inactivated after 100 minutes at 70°C, though it is relatively stable for 45 minutes at this temperature.

The optimum pH for enzyme activity is between 5.5 and 6.0, with reasonable activity between 5.0 and 7.0.

The enzyme preparation is relatively stable when stored at 5°C and 20°C up to 1 year with little loss of activity.

2.1.2 Technological purpose of the enzyme

Triacylglycerol lipase catalyses the hydrolysis of ester bonds of triacylglycerols (also called triglycerides), resulting in the formation of mono- and diacylglycerols, free fatty acids, and glycerol. This reaction is summarised in Figure 1 (taken from the reference listed).

Figure 22.3
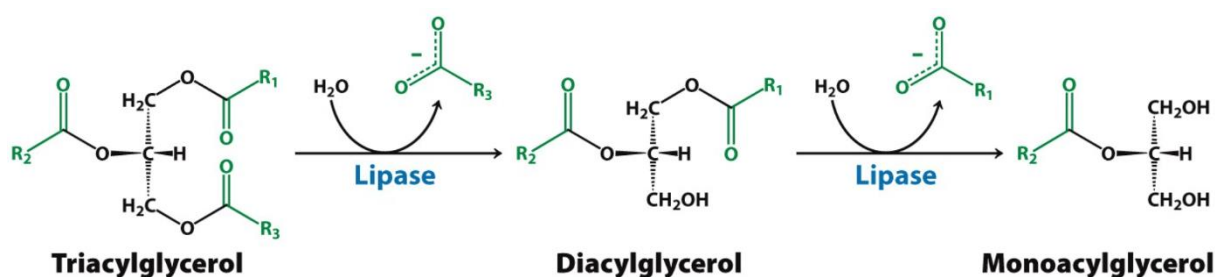*Biochemistry,* **Seventh Edition**
© 2012 W. H. Freeman and Company

Figure 1 Schematic indicating the reaction of triacylglycerol lipase

The technological purposes of the enzyme used in the baking and brewing industries are provided below.

Baking industry

Adding the enzyme to the dough for the production of baked goods improves the quality of

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[2] Chemical Abstracts Service.

these products. Wheat flour used in the baking industry typically contains around 2% lipids, half of which are usually non-polar lipids such as triglycerides, diglycerides, sterols and sterol esters, while the rest are polar lipids mainly containing galactolipids and phospholipids. Adding the enzyme to the dough modifies the non-polar lipids to produce more polar lipids such as lyso-phospholipids and lyso-galactolipids. Also the action of the enzyme on the non-polar lipids produces mono- and diglycerides and free fatty acids. The overall effect of the action of the enzyme on the dough is to improve the dough stability and handling properties. The polar lipids produced also lead to breads and baked goods with improved volume and a homogenous crumb structure with consistent pores.

When the enzyme is used to treat the dough for the manufacture of pasta and noodles it also produces more polar lipids which improves the quality of the final pasta or noodle product. This is turn results in lower cooking losses and improved eating quality.

In summary the benefits of using triacylglycerol lipase in the baking industry are:
- improved dough handling (extensibility and stability)
- improved dough structure and behaviour during baking
- increased uniformity and volume in baked products, with an improved crumb structure
- improved eating qualities of pasta and noodle products due to softer textural characteristics and improved appearance (whiteness, shine and smoothness)
- lower cooking losses for pasta and noodle products.

Brewing industry

Triacylglycerol lipase is used in the brewing process to improve mash separation and the fermentation process, specifically for non-malted cereals. In the mashing process the enzyme assists to reduce lipid concentration, which improves the separation of the liquor (the wort) from the mash solids. The fermentation step where the wort is fermented with yeast is also improved by the enzyme reducing the lipid (triglycerides) concentration where lipids can encapsulate the active yeast and so impede the fermentation.

In summary the benefits of using triacylglycerol lipase in the brewing industry are:
- increased flexibility in choice of raw materials (i.e. some proportion of non-malted cereals can be used)
- more efficient and more predictable mash separations
- higher extract yield from the mash
- potential for higher alcohol yield
- increase in filtration rate and so less use of filtration aids
- reduced potential for beer haze to form in the final packaged product.

2.1.3 Technological justification of the enzyme

There are a number of permitted triacylglycerol lipase enzymes from different microbial sources listed in Schedule 18 (in the tables to subsections S18—4(5) and 18—9(3)). The enzymes are listed in Schedule 18 as lipase, triacylglycerol. Permitting this enzyme provides the baking and brewing industries with an alternative commercial source of the enzyme. The applicant stated that the enzyme has superior tolerance to withstand mechanical shock during processing compared to the enzyme from different sources. Which enzyme companies use will depend on a range of commercial considerations including performance in the production of their products in their plants and economic factors.

The benefits of using the enzyme in the baking and brewing industries have been detailed in the previous section. The applicant provided details of its own studies supporting its claim that use of the enzyme does achieve these benefits.

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In the baking process the enzyme contributes to improved dough stability and handling properties. In practical terms for bread baking it increases the loaf volume and also the softness and softness retention with shelf life of the loaf. For the production of steamed buns use of the enzyme improves the appearance (whiteness, shine and smoothness) as determined by sensory evaluation. The bun size is also increased compared to the control that did not use the enzyme.

The use of triacylglycerol lipase in place of the enzyme mixture currently used (amylase, xylanase, glucanase, protease and pullulanase) for brewing with a high proportion of non-malted barley provides excellent functionality even up to 100% barley compared to 80% barley without the addition of triacylglycerol lipase. For 100% barley brewing, the addition of this enzyme to the existing enzyme mixture for the mash separation improves the wort filtration as well as the clarity of the wort. An increased filtration rate is noted after 5 minutes, while filtrate volume is approximately double the control after 30 minutes.

For 100% barley brewing, the extract after mashing is also improved when the enzyme is added compared to the control enzyme mixture. This is demonstrated by the analysis of the produced wort, with an increase in extractable sugars.

The enzyme is denatured when it is used as a processing aid either in the brewing or baking industries. In the brewing processes it undergoes a boiling step after it performs it technological purpose, while when it is used for baking it will be denatured due to the heat of baking.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme manufacturing process is composed of a fermentation process, a purification process, a formulation process and finally quality control of the finished product (see Figure 2).

Fermentation
The triacylglycerol lipase preparation is produced by submerged fed-batch pure culture fermentation of the GM *T. reesei*. This process is commonly used for the production of food-grade enzymes.

The fermentation process involves three steps, laboratory propagation of the culture (inoculation), seed fermentation and main fermentation.

Recovery
The recovery process is a multi-step operation designed to separate the enzyme from the microbial biomass and purify, concentrate, and stabilise and standardise the food enzyme preparation.
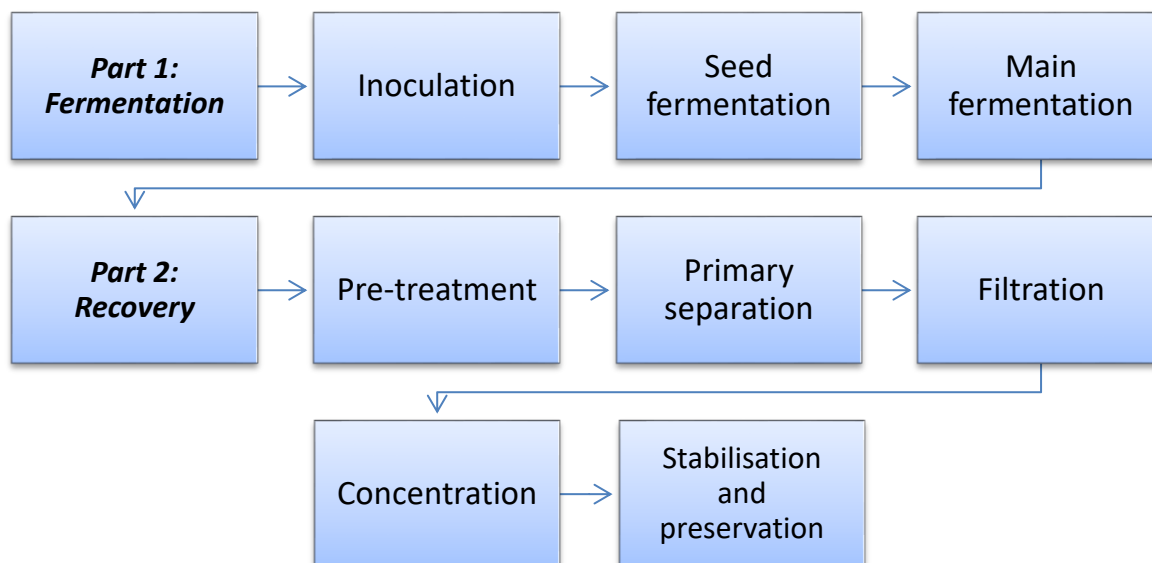
Figure 2: *Manufacturing process for triacylglycerol lipase preparation*

The enzyme preparation is manufactured in accordance with current Good Manufacturing Practices (cGMP).

The manufacturing process is such that the production microorganism, GM *T. reesei*, is removed during processing and is not detected in the commercial enzyme preparation.

The enzyme is standardised to ensure consistent enzyme activity and produced either as a liquid or a powder depending on how the enzyme preparation will be used.

2.2.2 Specifications

There are international specifications for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the Food Chemicals Codex (FCC) (USP, 2014). Both of these specification sources are primary sources listed in section S3—2 of the Australia New Zealand Food Standards Code (the Code). Enzyme preparations must meet these specifications.

Table 2 provides a comparison of three non-sequential representative batch analyses of the triacylglycerol lipase enzyme preparation with the international specifications established by JECFA and FCC, as well as those detailed in the Code (being section S3—4, as applicable).

Table 2: *Comparison of three representative samples of the commercial enzyme preparations with international enzyme specifications (JECFA and Food Chemicals Codex)*

Analysis	Enzyme batch analysis	Specifications		
		JECFA	Food Chemicals Codex	the Code
Lead (mg/kg)	<0.05, <0.05, <0.05	≤ 5	≤ 5	-
Arsenic (mg/kg)	<0.1, <0.1, <0.1	-	-	≤1
Cadmium (mg/kg)	<0.01, <0.01, <0.01	-	-	≤1
Mercury (mg/kg)	<0.01, <0.01, <0.01	-	-	≤1
Total coliforms (cfu/g)	<1, <1, <1	≤30	≤30	-
Salmonella (in 25 g)	ND[a], ND, ND	Absent	Negative	-
Enteropathic *E. coli* (in 25 g)	ND, ND, ND	Absent	-	-
Antimicrobial activity	ND, ND, ND	Absent	-	-
Production strain	ND, ND, ND	-	-	-
Mycotoxins	ND, ND, ND	No toxicological significant amounts	-	-

ND[a] = Not detected

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

2.3 Food technology conclusion

FSANZ concludes that the stated purpose of this enzyme preparation as a processing aid in the baking and brewing industries is clearly articulated in the application. The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The stated purpose is to hydrolyse triacylglycerols into mono- and diacylglycerols in the baking and brewing industries. The enzyme performs its technological purpose during the production and manufacture of foods after which it is inactivated thereby not performing a technological function in the final food. It is therefore appropriately categorised as a processing aid and not a food additive. The enzyme preparation meets international purity specifications.

3 Safety assessment

3.1 Objectives for safety assessment

The objectives of this safety assessment for triacylglycerol lipase are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by a genetically modified organism, as a processing aid. Specifically by considering the:

- history of use of the host and gene donor organisms
- characterisation of the genetic modification(s)
- safety of the enzyme.

3.2 History of use

3.2.1 Host organism

T. reesei was first isolated from canvas made from cotton, and 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 to Standard 1.3.3 of the Code permits the use of the following 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)

Aspergillus niger var. tubingensis

The gene sequence for the lipase 3 gene is from *Aspergillus niger var. tubingensis.* FSANZ has previously assessed the safety of *Aspergillus niger* for a number of enzymes used as processing aids. Schedule 18 to Standard 1.3.3 of the Code permits the use of the following enzymes derived from *A. niger*: α-amylase, α-arabinofuranosidase, asparaginase, aspergillopepsin I, aspergillopepsin II, catalase, cellulase, chymosin. endo-1,4-beta-xylanase, endo-arabinase, endo-protease, β-fructofuranosidase, α-galactosidase, β-galactosidase, β-glucanase, glucoamylase, glucose oxidase, α-glucosidase, β-glucosidase, hemicellulase multicomponent enzyme, inulinase, lipase triacylglycerol, lipase triacylglycerol, protein engineered variant, lysophospholipase, pectin lyase, pectinesterase, phospholipase A2, 3-phytase, polygalacturonase or pectinase multicomponent enzyme, transglucosidase. The NCBI Protein BLAST search of the lipase 3 gene sequence returned 98% identity to *A. niger* lipase.

Aspergillus nidulans

The gene sequence for acetamidase, used as the selection marker for positive transformants, was initially isolated from *Aspergillus nidulans* (Kelly and Hynes, 1985). The majority of *A. nidulans* strains have been classed at the Biosafety Level 1, however some strains have been associated with opportunistic infections in immunocompromised individuals (Gabrielli et al, 2014; Henriet et al. 2012). As the gene sequence has been manipulated through standard DNA cloning methods subsequent to the original isolation from the donor organism, extraneous material from *A. nidulans* would not be carried across to the enzyme production organism. The acetamidase protein is not secreted from the production strain, so will not be present in the purified lipase product nor will be present in the final food.

3.3 Characterisation of the genetic modification(s)

3.3.1   Description of DNA to be introduced and method of transformation

The production strain (Morph Lip3) was derived from a parental strain, RL-P37 (Sheir-Neiss & Montenecourt 1984) that itself originated via classical mutagenesis from strain QM6a and has previously been used by the applicant to manufacture commercial cellulase and xylanase preparations. QM6a is a well-characterised, single wild-type isolate from the Solomon Islands and has been used to develop numerous strains for industrial purposes (see e.g. Seidl et al 2009).

RL-P37 then underwent a complex series of genetic modifications via protoplast-mediated transformation procedures (see e.g. Penttila *et al.*, 1987; Gruber *et al.,* 1990; Smith *et al.*, 1991), using protoplasts prepared from mycelia (Penttilä et al, 1987; Karhunen et al, 1993), to reduce cellulytic activities by inactivating several key genes. As a result of this inactivation, the production strain is unable to survive in the natural environment because it is unable to use cellulose as a carbon source. Facilitation of selecting desired transformants during the development of the final strain was achieved via a system that makes use of the recyclable cassette which contains the *T. reesei pyr4* gene encoding orotidine 5′-phosphate carboxylase. This marker gene can be bi-directionally selected and the *pyr4* cassette can be re-used in multiple rounds of genetic transformation (Seidl and Seiboth 2010). The final strain does not contain this gene.

In the final stage of transformation the *T. reesei* strain Morph Lip3 was produced by spore electroporation to integrate a PCR-purified linear DNA fragment expression cassette (i.e. free from any vector-derived sequences) isolated from a plasmid and containing two coding regions. The first coding region, was driven by the strong native *T. reesei* promoter from the cellobiohydrolase 1 (*cbh1*) gene and contained the lipase 3 (*lip3*) gene (with signal sequence) from *Aspergillus niger var. tubingensis* with a *T. reesei cbh1* terminator. The second coding region comprised the acetamidase gene (*amdS)* from *A. nidulans* (Kelly and Hynes, 1985), with its own promoter and terminator. This second gene allows for selection of transformants on acetamide-containing media and has been widely used as a selection marker in fungal transformations in the past. In addition, the cassette also contained two short (25 bp) recombination site cloning remnants.

3.3.2   Characterisation of inserted DNA

At all stages of the transformations, Southern blot analysis with appropriate controls (and following restriction enzyme digestion and polymerase chain reaction) was undertaken to ensure no extraneous DNA sequences had been incorporated. At the final stage, Southern analysis of the Morph Lip3 production strain showed that a) no vector DNA was present (and hence, the ampicillin resistance in the plasmid backbone – used for passage of the cloning vector *in E. coli* - was not present) and b) more than one intact copy of the expression cassette had been incorporated into the genome. Further to this, next generation sequencing was used, in conjunction with mapping to a *T. reesei* genome database, to identify the site of integration of the expression cassette in the genome and estimate the number of copies of the *lip3* gene integrated into the genome.

3.3.3   Genetic stability of the inserted gene

T. reesei strains in general, are widely used in biotechnological processes because of their known stability.

The genetic stability of the inserted gene was demonstrated by genome sequencing. Broth samples were taken prior to and after three fermentations in 14 L fermenters mimicking

commercial fermentation conditions. Samples were then used for genomic DNA extraction and next generation sequencing, which covered over 99% of the genome. A complex integration site for lipase expression site was determined, and no change was observed between samples prior to and after fermentation. The results demonstrate that the insertion cassette had been stably maintained through generations during the fermentation process. In addition, the applicant states that the production strain proved to be 100% stable after at least 60 generations of fermentation, as judged by lipase production.

3.4 Safety of triacylglycerol lipase

3.4.1 History of safe use of the enzyme

FSANZ has approved the use of triacylglycerol lipase as a processing aid, from both animal origin (bovine, ovine and porcine) and fungal sources (A264, A402, A435, A516, A517, A519, A569, A1036 and A1130). Some of the approvals pre-date the establishment of the current version of the Code, indicating that the use of this enzyme as a processing aid in food spans more than 30 years. FSANZ is not aware of any reports of adverse effects resulting from its use as a food processing aid.

The applicant has indicated that triacylglycerol lipase from *T. reesei* strain Morph Lip3 has been used for pizza, bread and other bakery applications in the US and in bakery products in India since 2017. The applicant is not aware of any adverse events reported since this form of the enzyme has been in commercial use in these countries.

3.4.2 Toxicity studies of the enzyme

Reports of four toxicity studies with the triacylglycerol lipase enzyme have been submitted: an acute oral toxicity study and a 90-day repeated dose oral toxicity in rats, a bacterial reverse mutation assay and an *in vitro* chromosomal aberration test conducted with human lymphocytes. In these studies the enzyme preparation tested was an ultra-filtered concentrate containing 178.4 mg total protein/mL, lot number 20088167. The enzyme total organic solids (TOS) content was 0.77 mg per mg total protein.

3.4.2.1 Studies in experimental animals

Acute oral toxicity study in rats (MB Research Laboratories 2009) Regulatory status: GLP; Conducted according to OECD Test Guideline (TG) 425

Initially, a single female Wistar albino rat was administered lipase by oral gavage at a dose of 2000 mg/kg bw. As the animal survived, four additional females were dosed at 2000 mg/kg bw. Rats were observed for signs of toxicological or pharmacological effects at 15 minutes, 1, 2 and 4 hours post-dosing and once daily for 14 days. Body weights were recorded prior to testing and on study days 7 and 14. Additional body weights for one animal were recorded on days 15 to 19. All animals were killed at study termination and examined for gross pathology.

All animals survived to the end of the study and no clinical signs of toxicity were observed. Body weight changes were normal in 3/5 animals. Two animals lost weight between days 7 and 14 (bodyweight losses of ~ 4% and 1%). The animal with the greater body weight loss was observed for an additional 5 days, during which time fluctuations in body weight were observed. No pathological changes were observed at necropsy.

The LD_{50} of lipase was greater than 2000 mg/kg bw under the conditions of this study.

90-day repeated dose oral toxicity study in rats (Harlan Laboratories 2010) Regulatory

status: GLP; Conducted according to OECD TG 408

Lipase was administered by oral gavage to Wistar rats (10/sex/group) at doses of 0, 53.5, 80.3 and 160.6 mg/kg bw/day total protein for a period of 91/92 days. These doses corresponded to TOS doses of 0, 41.02, 61.57 and 123.15 mg/kg bw/day, respectively, or 0, 32,390, 48,610 and 97,225 lipase units (LIPU) per kg bw/day. The vehicle control was 0.9% saline solution. Clinical signs, cage-side observations, detailed behavioural observations, food consumption and body weights were recorded periodically throughout the acclimatisation and treatment periods. Ophthalmologic examinations were performed in all animals during acclimatisation and in animals of the control and high dose groups during week 13. A functional observational battery, grip strength and locomotor activity were also assessed during week 13. At the end of the dosing period animals were fasted for approximately 18 hours and blood and urine samples were collected. All animals were killed, necropsied and examined post mortem. Histopathological examination was conducted on organs and tissues from all control and high dose animals, and gross lesions were assessed for all animals.

One control female was killed for humane reasons on day 57 of treatment, due to the presence of a malignant, lymphoblastic lymphoma. No mortality occurred in the low dose group. In the mid dose group one male was found dead on day 46 and two females were found dead on days 31 and 45. One high dose female was found dead on day 39. Pathology findings for animals that died (e.g. blood aspiration, dark red discolouration of lungs, alveolar edema) were consistent with gavage errors.

No test item-related clinical signs of toxicity were observed at any dose during the course of the study, including in the functional observational battery. In males, significantly higher hind-limb grip strength was observed at all doses of Lipase 3, but in the absence of a dose-response relationship or similar changes in fore-limb grip strength these differences were considered to be incidental. No test item-related differences in locomotor activity were noted at any dose level. There were no test item-related differences in food consumption, body weight or body weight gain, ophthalmological examination, haematology, clinical chemistry or urinalysis parameters. A small number of statistically significant differences in mean absolute and/or relative organ weights compared with controls were observed, but these changes were not considered to be related to the test item based on a lack of a dose-response relationship and the absence of accompanying histopathologic changes. There were no gross or microscopic lesions that could be attributed to treatment with the test item.

It was concluded that the no observed adverse effect level (NOAEL) of the lipase in this study was 160.6 mg/kg bw/day total protein, the highest dose tested. This corresponds to 123.15 mg/kg bw/day TOS or 97,225 mg/kg bw/day LIPU.

3.4.3 Genotoxicity

Bacterial reverse mutation assay (Harlan Laboratories 2009a) Regulatory status: GLP; Conducted according to OECD TG 471

The mutagenic potential of lipase was assessed in a bacterial reverse mutation assay using the plate incorporation (Experiment I) and pre-incubation (Experiment II) methods. Bacterial strains used were *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and the *Escherichia coli* strain WP2 uvrA. The assays were performed in the presence and absence of metabolic activation (S9 mix). The vehicle was water, and vehicle and untreated controls were included. Positive controls in the absence of metabolic activation were sodium azide (for strains TA 1535 and TA 100), 4-nitro-o-phenylene-diamine (TA 1537 and TA 98) and methyl methane sulfonate (WP2 uvrA). In the presence of metabolic activation the positive control was 2-aminoanthracene. Lipase was tested at 8 concentrations ranging from

3 – 5000 µg/plate and each concentration, including the controls, was tested in triplicate.

Plates incubated with lipase showed normal background growth at all doses with and without metabolic activation in both experiments. No substantial increase in the number of revertant colonies was observed in any of the test strains following treatment with lipase in the presence or absence of S9. There was also no concentration-related tendency for higher mutation rates below the levels considered of biological relevance. Negative and positive controls produced the expected responses, confirming the validity of the test system.

Lipase was not mutagenic under the conditions of this study.

Chromosome aberration test in human lymphocytes (Bohnenberger 2009) Regulatory status: GLP; conducted according to OECD TG 473

Lipase was tested for its ability to induce structural chromosome aberrations *in vitro* using cultured human lymphocytes. The test was performed in the presence and absence of metabolic activation (S9 mix), and the vehicle and negative control was water. Positive controls were cyclophosphamide and ethylmethane sulfonate in the experiments with and without metabolic activation, respectively. Two experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9, while in the Experiment II the exposure period was 4 hours with S9 and 22 hours without S9. Both experiments were performed 40 hours after mitogenic stimulation of lymphocytes with phytohaemagglutinin. Chromosomes were prepared 22 hours after the start of treatment with lipase, and colcemid was added to the cultures 3 hours before harvesting. All experiments included two parallel cultures at each test concentration, and 100 metaphases per culture were scored for chromosomal aberrations. An exception was the positive control in experiment II with S9 mix, where 50 metaphases per culture were scored. The mitotic index of the cultures was assessed by counting 1000 cells per culture. Concentrations of lipase ranged from 32.5 – 5000 µg/mL and for all experiments the three highest concentrations (1632.7, 2857.1 and 5000 µg/mL) were evaluated for chromosomal aberrations.

Lipase was not cytotoxic in the presence or absence of metabolic activation in either experiment. No significant increases in the number of cells with structural chromosomal aberrations were observed in either experiment compared with the vehicle controls, with or without metabolic activation. The rates of chromosomal aberrations in treated cells were also within the range of the laboratory's historical negative control data. There was no evidence of an increase in polyploid metaphases after treatment with the test item, compared with negative controls. Both positive controls induced significant increases in the number of cells with structural chromosomal aberrations, confirming the validity of the test system.

Lipase did not induce structural chromosomal aberrations under the conditions of this study.

3.4.4 Bioinformatic analysis for potential toxicity

The applicant provided results from *in silico* analyses comparing the amino acid sequence for the lipase protein with known toxins and antinutrients using the UniProtKB annotated Protein Knowledge database. The vast majority of hits were with lipases, and none of the top 1000 database hits were annotated as toxins or venom proteins.

3.4.5 Potential for allergenicity

The applicant provided the results of an *in silico* analysis comparing the mature lipase amino acid sequence to known allergenic proteins using the Food Allergy Research and Resource Program (University of Nebraska) AllergenOnline database. The searches were performed on 21 September 2017.

Three types of analyses were performed for this comparison:

- A FASTA sliding window search for contiguous 80 amino acid stretches within the lipase sequence with greater than 35% identity to known allergens in the database
- A FASTA search for full-length sequence alignments, with identity matches greater than 50% indicating possible cross-reactivity, using an E-value threshold of 0.1
- A search for exact matches of 8 contiguous amino acid sequences with known allergens.

No homology was found between the lipase amino acid sequence and any known allergenic proteins.

The applicant has indicated that the final dry products for bakery applications can be spray-dried on potato or wheat starch. Wheat is considered to be a major food allergen, however as bakery products can be produced using wheat no additional allergens are introduced into the final food.

Nitrogen sources used in the fermentation medium during manufacture of the enzyme may include soy protein, and glucose and sorbitol products derived from wheat or other gluten-containing cereals. Therefore the enzyme preparation may contain traces of cereals or soy. The applicant has estimated that the highest amount of soy protein or cereal protein in the final food would be 2-3 ppb and 5 ppb, respectively.

3.4.6 Approvals by other regulatory agencies

In the USA, a response from the Food and Drug Administration (FDA) to a generally recognised as safe (GRAS) notification on the applicant's enzyme preparation (GRN 808) is pending. No approvals by other regulatory agencies have been identified.

4 Conclusions

There are no public health and safety concerns for the general population associated with the use of triacylglycerol lipase from *T. reesei* strain Morph Lip3 as a food processing aid.

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 lipase gene has been shown to be stably inherited.

The enzyme showed no evidence of genotoxicity in a bacterial reverse mutation assay or a chromosomal aberration assay in human lymphocytes. In a 90-day oral gavage study in rats, the NOAEL was the highest dose tested, 160.6 mg/kg bw/day total protein, which is equivalent to 123.15 mg/kg bw/day TOS. The applicant's estimated theoretical maximum daily intake (TMDI) based on the proposed uses is 0.410 mg/kg bw/day TOS. From these values, the Margin of Exposure is approximately 300.

Bioinformatic data indicate a lack of homology with known toxins or allergens, and the enzyme is unlikely to pose an allergenicity concern. The applicant has indicated that enzyme products used for bakery production may contain wheat. The enzyme product may also contain traces of soy and gluten-containing cereals.

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. A dietary exposure assessment was therefore not required.

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