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[75-19]

Supporting document 1

Risk and technical assessment report – Application A1162

Triacylglycerol lipase from *Trichoderma reesei* as a processing aid (enzyme)

Executive summary

The purpose of the application is to seek amendment of Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include the enzyme triacylglycerol lipase (EC 3.1.1.3) from a genetically modified strain of *Trichoderma reesei* as a processing aid in baking and other cereal-based processes.

FSANZ has assessed the information on this triacylglycerol lipase provided with the application, and has determined that the data provided are adequate for this assessment.

The stated technological purpose of the triacylglycerol lipase, namely, for use as a processing aid in baking and other cereal-based processes, is clearly articulated in the application. The evidence presented to support the proposed use of the enzyme 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 enzyme meets international purity specifications.

The production organism *T. reesei* is not pathogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Molecular characterisation of the production strain has confirmed the sequence of the inserted DNA is as expected and has not undergone any rearrangement, and the introduced DNA is stably inherited.

Triacylglycerol lipase from *T. reesei* was not genotoxic *in vitro* and did not cause adverse effects in a subchronic toxicity study in rats. The no observed adverse effect level (NOAEL) in the subchronic toxicity study was the highest dose tested, 1000 mg/kg bw/day on a total organic solids (TOS) basis. This is more than 11,000-fold higher than the applicant's estimate of an individual's theoretical maximal daily intake (0.09 mg TOS/kg bw/day) based on the proposed uses.

Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens and is unlikely to pose an allergenicity concern. However, the enzyme preparation contains wheat flour as a carrier. As wheat is a major food allergen, risk management measures are indicated to protect wheat-allergic individuals.

In the absence of any identifiable hazard an acceptable daily intake 'not specified' is appropriate. A dietary exposure assessment was therefore not required.

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1 Introduction

AB Enzymes GmbH has made an application to FSANZ seeking permission to use the enzyme triacylglycerol lipase (EC 3.1.1.3) from a genetically modified strain of *Trichoderma reesei* as a processing aid for use in baking and other cereal-based processes. Specifically, the enzyme would be used in baking processes, for foods such as bread, biscuits, tortillas, cakes, steamed bread and croissants, and in other cereal-based processes, for foods such as pastas and noodles.

AB Enzymes GmbH is an industrial biotechnology company that develops, manufactures and supplies enzyme preparations for industrial applications. The enzyme preparation that is the subject of this application has been authorised for use in France (April 2017) and the United States (US GRAS no objection letter GRN000631). A dossier on the enzyme has also been submitted to the European Food Safety Authority (EFSA) (EFSA-Q-2016-00212) and is currently under review.

There are a number of permissions for triacylglycerol lipase in the Australia New Zealand Food Standards Code (the Code) (FSANZ 2018). However, there is no permission for use of this enzyme sourced from a genetically modified strain of *T. reesei*, containing the gene for triacylglycerol lipase isolated from *Fusarium oxysporum*. Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

1.1 Objectives of the assessment

The objectives of this technical, safety and risk 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 protein, 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 protein.

2 Food technology assessment

2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

Information concerning the identity of the enzyme from the application has been verified using an appropriate enzyme nomenclature reference (IUBMB 2017).

Systematic name: Triacylglycerol acylhydrolase

Accepted IUBMB¹ name: Triacylglycerol lipase

¹ International Union of Biochemistry and Molecular Biology.

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 lipase; liver lipase; hepatic monoacylglycerol acyltransferase

IUBMB enzyme nomenclature: EC 3.1.1.3

Host microorganism: *Trichoderma reesei*

Gene donor microorganism: *Fusarium oxysporum*

CAS² registry number: 9001-62-1

Reaction: Triacylglycerol + H₂O = diacylglycerol + carboxylate

2.1.2 Technological purpose of the enzyme

The enzyme is to be used as a processing aid in baking and other cereal-based processes. Triacylglycerol lipase catalyses the hydrolysis of ester bonds of triacylglycerols (or triglycerides), resulting in the formation of mono- and diacylglycerols, free fatty acids, and glycerol. Figure 1 below shows the hydrolysis of a triacylglycerol to form a diacylglycerol and a free fatty acid.



Source: https://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/enzymes/GetPage.pl?ec_number=3.1.1.3

Figure 1 Representation of hydrolysis reaction catalysed by triacylglycerol lipase

During mixing of the dough, triglycerides (lipids) in wheat flour may become bound or trapped within the gluten³ component of the dough. The action of triacylglycerol lipase on triglycerides assists during the baking of bread, biscuits, cakes etc. by:

- producing a greater proportion of polar lipids, which align at the interface of gas cells and help increase their stability. This assists with gas retention and volume of the baked product

² Chemical Abstracts Service.

³ Gluten is a protein present in cereal grains like wheat (and their flours), and is responsible for the elastic texture of the dough.

- creating monoacyl-glycerol, which interacts with amylose⁴ to form complexes that enhance the dough's structure and behaviour during baking.

For other cereal-based processes including the production of pasta and noodles, the action of the enzyme assists primarily by creating hydrolysis products that form complexes with amylose which:

- help maintain the protein network of the pasta during cooking. This also plays an important role in reducing the porosity of noodles and oil uptake during frying
- inhibit the swelling of starch and leakage of amylose during cooking, resulting in a pasta product which has a firmer texture and smoother surface, and is less sticky and more elastic.

The enzyme manufacturing process results in a concentrated triacylglycerol lipase solution, which is formulated, packaged and sold as a powdered enzyme preparation. The composition and physical properties of the triacylglycerol lipase enzyme preparation, sold under the commercial name of Veron® Hyperbake ST, are presented below in Table 1 and Table 2 respectively.

Table 1 *Triacylglycerol lipase enzyme preparation composition*

Composition of Veron® Hyperbake ST	
Lipase	8.8%
Sunflower oil	0.4%
Wheat flour	90.8%

Table 2 *Triacylglycerol lipase enzyme preparation physical properties*

Physical properties of Veron® Hyperbake ST	
pH value	7-9
Density	1.00-1.10 g/ml
Appearance	Light beige colour with characteristic, aromatic odour
Min. enzyme activity	22 500 activity units

Whilst the enzyme preparation contains predominantly triacylglycerol lipase activity, there are also small amounts of other enzymatic side activities, which are known to be produced by all microorganisms including *T. reesei* which, for this source, are mainly endoglucanase and xylanase. Supporting documentation shows the levels of these side activities is small, and the assays used to measure them were provided with the application. The documentation provided sufficient evidence that the side activities present in the triacylglycerol lipase solution are not relevant to this application, mainly due to the small quantities involved, especially when compared to enzyme preparations that have been standardised for those specific activities.

Use of commercial enzyme preparations are typically in accordance with Good

⁴ Amylose is one of the two main components of starch, made up of long polysaccharide chains.

Manufacturing Practice (GMP), whereby use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The recommended use level of Veron® Hyperbake ST is 0.5-3 g/100 kg of flour. The technical information sheet provided with the application provides advice that the optimum use level should be determined by means of baking tests. Food manufacturers will thus fine-tune the level of enzyme used on a case-by-case basis, founded on the desired effect and their individual process conditions.

2.1.3 Technological justification for the enzyme

As mentioned above, triacylglycerol lipase present in baking and other cereal-based processes can have a positive effect on the interactions between the constituents of the dough, i.e. gluten proteins and lipids, starch and lipids as well as gluten and starch. Triacylglycerol lipase supplements endogenous cereal grain lipases and is added as a dough improver, which manifests as increased bread volume, more uniform crumb mixture and air cell size and lesser tendency to stale, without influencing rheological (mixing) properties of the dough (Damodaran et al, 2008).

The technological justification for using triacylglycerol lipase in baking, as indicated by the application, is that it breaks down triglycerides to produce benefits such as:

- facilitating the handling of the dough, through its positive influence on the interactions of the dough's various constituents
- improving dough stability and strength which results in an increase in processing tolerance
- improving the dough's structure and performance during the baking steps, to increase bread volume
- regulating batter viscosity, specifically in producing waffles, pancakes and biscuits.

The technical justification for using triacylglycerol lipase in other cereal-based process is similar to that for baking. In addition to facilitating dough handling and improving dough stability/strength, other benefits include:

- reducing cracking in dried noodles
- reducing oil uptake during frying
- improving dough emulsification (Fernandes 2010)
- reducing surface stickiness and increasing firmness of cooked noodles, which is associated with reduced leakage of starch, perhaps through complexing with fatty acids and lysoglycerolipids (Gulia et al, 2013; Damodaran et al, 2008).

Moreover, the triacylglycerol lipase that is the subject of this application is further technologically justified because it has been found to have an increased tolerance to mechanical shock during processing. Specifically, the applicant provided information regarding a benchmarking exercise (baking test) against two competitors' products. The results of the baking test indicated that the product in which the enzyme was used showed improved structural dough stability. It was assumed that this is because of the created ratio of converted polar lipids in the dough in which this enzyme was used is different and beneficial towards dough stability.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The enzyme is produced by controlled submerged fermentation of the genetically modified production strain of *T. reesei*.

The production process comprises three main process steps: fermentation, recovery and preparation of the final product. Production begins with a pure culture of *T. reesei*, which is added to a shake flask containing fermentation medium. When sufficient biomass is obtained, the contents of the shake flasks are used to inoculate the seed fermentor. At the end of fermentation, the inoculum is then transferred to the main fermenter containing fermentation medium. Biosynthesis of triacylglycerol lipase occurs in the main fermenter under well-defined process conditions governing the pH, temperature, time and mixing.

Once the fermentation is complete, the recovery process is initiated. The fermentation broth undergoes separation and concentration steps. The separation step involves removing the biomass from the fermentation medium containing the desired enzyme protein. This process is performed at defined pH and temperatures to minimise loss of enzyme activity. The concentration step ensures that the enzyme solution achieves the desired enzyme activity prior to formulation.

After concentration, the enzyme is run through polish and germ filtration, to remove residual cells of the production strain, eliminate any microbial contamination, and remove any other insoluble substances that may still be present. The resultant concentrated enzyme solution is ready for formulating into a dry food enzyme preparation (see section 2.1.2) and then packaged and sold commercially.

Materials used in the fermentation and recovery process are standard, food grade quality materials that meet quality standards and relevant specifications, including those outlined in Food Chemicals Codex (10th edition) (2016) that require that substances such as processing aids used to produce enzyme preparations be acceptable for general use in foods. Furthermore, production of the enzyme is in accordance with current GMP and Hazard Analysis and Critical Control Points (HACCP) principles, and in compliance with the food hygiene regulations that apply in the country of manufacture (e.g. [Regulation \(EC\) No. 852/2004](#) on the hygiene of foodstuffs⁵). The applicant has provided quality certificates demonstrating their adherence to these practices and principles.

The main steps of the manufacturing process are shown in Figure 2 below taken from the application.

⁵ <https://www.ecolex.org/details/legislation/regulation-ec-no-8522004-of-the-european-parliament-and-of-the-council-on-the-hygiene-of-foodstuffs-lex-faoc063426/>

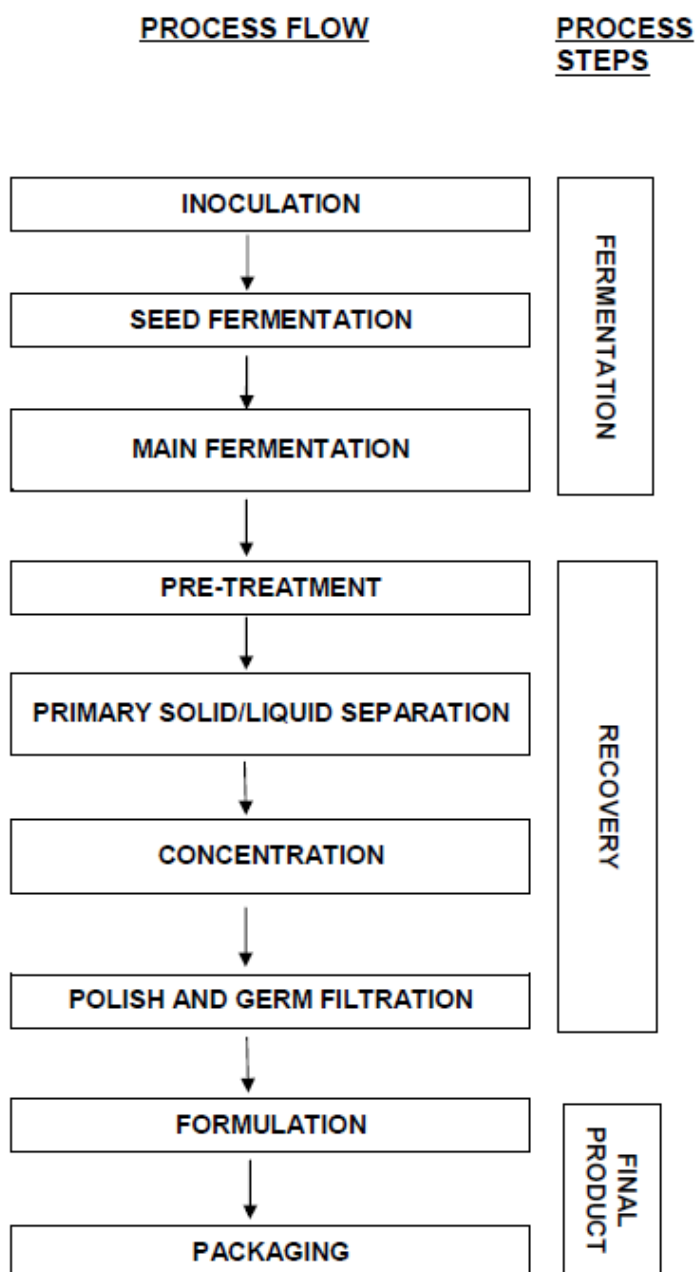


Figure 2 Production process of triacylglycerol lipase food enzyme from fermentation

2.2.2 Specifications

There are international specifications for enzymes used in the production of food. These have been detailed in the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Compendium of Food Additive Specifications (FAO/WHO 2016) and the United States Pharmacopeial Convention (2016) Food Chemicals Codex (10th edition). These primary sources of specifications are listed in section S3—2 of the Code. Enzymes need to meet these enzyme specifications.

Table 3 provides a comparison of batch analysis of three dry enzyme concentrates of triacylglycerol lipase from the genetically modified *T. reesei* with the international

specifications established by JECFA and Food Chemicals Codex, as well as those in the Code, as applicable.

Table 3 Analysis of AB Enzymes triacylglycerol lipase compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Analysis	AB Enzymes batch analysis (3 different batches)	Specifications		
		JECFA	Food Chemicals Codex	Australia New Zealand Food Standards Code (section S3—4)
Lead (mg/kg)	<0.05 0.06 <0.05	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.5 <0.5 <0.5	-	-	≤1
Cadmium (mg/kg)	<0.05 0.05 <0.05	-	-	≤1
Mercury (mg/kg)	<0.1 <0.1 <0.05	-	-	≤1
Coliforms (cfu/g)	<10 <10 <10	≤30	≤30	-
<i>Salmonella</i> (in 25 g)	ND ND ND	Absent	Negative	-
<i>E. coli</i> (in 25 g)	ND ND ND	Absent	-	-
Antimicrobial activity	ND ND ND	Absent	-	-
Mycotoxins	<10	No toxicologically significant amounts	-	-
T2-toxin	<20			
HT-2-toxin	<10			

*ND – not detected

Based on the comparison to the above specifications, the dry enzyme concentrate meets international specifications for enzymes used in the production of food.

2.2.3 Stability

Triacylglycerol lipase has optimal activity between pH 7-8. Its optimum activity is achieved

around 30°C, with its activity dropping above 30°C. The enzyme is inactivated at 85°C (after one minute). The enzyme is denatured by heat during a boiling/steaming or baking step.

The powdered enzyme preparation will lose less than 10% of enzyme activity within one year, when stored in a dry place at room temperature.

2.3 Food technology conclusion

The stated purpose of this triacylglycerol lipase enzyme, namely, for use as a processing aid for baking and other cereal-based processes, is clearly articulated in the application. This enzyme will be used to hydrolyse triacylglycerols into mono- and diacylglycerols, free fatty acids, and glycerol, resulting in doughs and batters that have improved properties and, as such, higher quality baked products.

AB Enzymes' triacylglycerol lipase is sourced from a genetically modified strain of *T. reesei*. Compared with other enzyme products on the market, this enzyme has been found to have an increased tolerance mechanical shock during processing.

The evidence presented to support the proposed use of the enzyme 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 enzyme meets international purity specifications.

3 Safety assessment

3.1 History of use

3.1.1 Host organism

T. reesei is a hypercellulolytic fungus commonly found in soil. The initial isolate came from deteriorating clothing and tent material found in the Solomon Islands after World War II. The isolate QM6a has been registered with the American Type Culture Collection (ATCC 13631) and has been classed as a Biosafety Level 1 organism, based on the [United States Public Health Service Guidelines](#)⁶. This fungus is not considered pathogenic to humans.

Due to the secretion of a range of cellulolytic enzymes, this fungus has been used since the 1980s for the industrial production of cellulase, hemicellulase, beta-glucanase, pectinase and xylanase for a range of industries including food (Nevalainen and Peterson, 2014; Paloheimo et al, 2016; FSANZ 2016). This organism therefore has a long history of safe use for the production of food grade enzymes.

Prior to generation of the final production strain (RF10625) outlined in Figure 3, the host organism was modified using a range of conventional mutagenesis (A) and genetic modification (B) steps that are routinely used to optimise organisms for industrial use. These changes have been characterised and include silencing of unnecessary proteins that would impact the yield and purity of a protein-of-interest and to increase the ability and efficiency to transform the host. In the process of optimisation, two intermediate organisms were generated, which FSANZ has called IO-1 an IO-2.

⁶ <https://www.cdc.gov/biosafety/publications/bmbl5/index.htm>

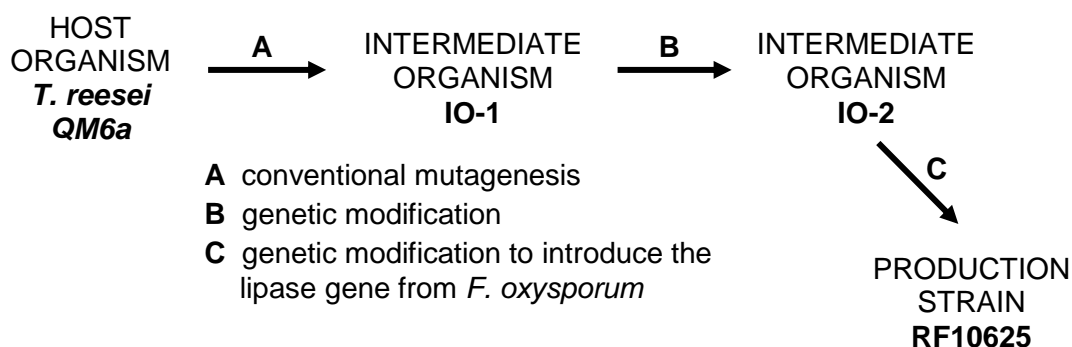


Figure 3 Outline of the production strain development pathway

The intermediate strain IO-1 derived from QM6a has been characterised by the Westerdijk Fungal Biodiversity Institute (CBS) in the Netherlands. The characterisation was based on DNA sequence analysis and confirmed the taxonomy of the organism as *T. reesei*.

3.1.2 Gene donor organism(s)

Fusarium oxysporum

F. oxysporum are filamentous fungi, commonly found in soil. Although many strains have been classed as Biosafety Level 1 organisms, there are several phyto-pathogenic strains that have been associated with opportunist infections in immunocompromised individuals (Nucci and Anaissie, 2007). The gene sequence for the triacylglycerol lipase enzyme was based on the gene from *F. oxysporum* (accession number CAB69359). The gene was chemically synthesised, to allow modification of the nucleotide sequence to allow codon optimisation. These modifications do not result in changes to the encoded peptide sequence.

Aspergillus nidulans

The gene sequence for acetamidase, used as the selection marker for positive transformants, was initially isolated from *Aspergillus nidulans* VH1-TRSX6 (Kelly and Hynes, 1985; Penttila et al, 1987). 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; Sadarangani et al, 2015). 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.

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of DNA to be introduced and method of transformation

A *T. reesei* strain IO-2 was transformed by a protoplast-mediated transformation procedure, using protoplasts prepared from hyphae (Penttila et al, 1987; Karhunen et al, 1993; Li et al, 2017). A purified linear DNA fragment encompassing two gene cassettes was included in the transformation mix to create the production strain. The main expression cassette (Figure 4) contained the gene for triacylglycerol lipase from *F. oxysporum* under the control of the well characterised promoter and terminator sequences from *T. reesei*. Within the lipase gene sequence there is the natural signal peptide sequence, which ensures the enzyme is secreted. No backbone vector sequences were included in the transformation reaction.



Figure 4 The triacylglycerol lipase gene cassette

A second gene cassette was also located in the DNA fragment, encoding for the acetamidase gene (*amdS*) from *A. nidulans*. This gene is not an antibiotic resistance gene but allows for selection of transformants on acetamide-containing media.

3.2.2 Characterisation of inserted DNA

Southern blotting was performed on genomic DNA extracted from the production and parental host strain and digested with various restriction enzymes. Hybridisation with a probe that encompassed the complete DNA fragment introduced into the production strain showed that more than one copy of the complete insert has been randomly integrated into the host's genome, and one tandem integration was identified. Hybridisation with a probe that encompassed just the lipase gene confirmed that there were multiple integration events and a tandem insertion. No hybridisation was observed in the host strain for either the full-length insert probe or the lipase gene probe.

In order to confirm absence of the plasmid backbone sequence, hybridisation with a probe specific to the plasmid backbone sequence showed no binding in either the production strain or host. However binding was observed to a vector-only control sample.

3.2.3 Genetic stability of the inserted gene

Southern blotting was performed on genomic DNA extracted from the production strain cultured for ten generations in media without acetamide. The DNA was digested with various restriction enzymes and hybridisation was performed with a probe that encompassed the complete DNA fragment introduced into the production strain. The results showed that expression of the lipase gene was consistent across generations, indicating the lipase gene was stably incorporated into the production strain.

3.3 Safety of triacylglycerol lipase

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to cause adverse health effects, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al, 2008). Furthermore, proteins perform a wide range of functions in humans. To encompass this range of type and function, the safety assessment of the novel protein must consider if there is a history of safe use, are there any potential toxic, anti-nutrient or allergenic effects and is the protein susceptible to digestion.

3.3.1 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.

3.3.2 Bioinformatic analysis for potential allergenicity

The applicant has provided the results of an *in silico* analysis comparing the mature triacylglycerol lipase amino acid sequence inserted into *T. reesei* to known allergenic proteins in the Food Allergy Research and Resource Program dataset, which is available through [AllergenOnline](http://www.allergenonline.org)⁷ (University of Nebraska) and the [Allergen Database for Food Safety](http://allergen.nihs.go.jp/ADFS/)⁸, administered by the Japanese National Institute of Health Sciences. The searches were performed on 26 February 2015.

Four types of analyses were performed for this comparison:

- a) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids within the triacylglycerol lipase sequence to the database entries. Matches were identified if there was greater than 35% homology (Pearson and Lipman, 1988).
- b) 8-mer exact match search – A FASTA alignment was performed comparing contiguous 8 amino acids within the triacylglycerol lipase sequence to the database entries (FAO, 2001). Matches were identified if there was 100% homology.
- c) Full length sequence search - a BLAST search was performed comparing the whole sequence to the database entries. Significant homology was determined when there was more than 50% similarity between the query protein and database entry, with the E-value threshold conservatively set at 1×10^{-1} (1e-1).
- d) Motif-based search - a BLAST search was performed comparing the whole sequence to the database entries (Stadler and Stadler, 2003). Significant homology was determined when a score greater than 8.5 was achieved or high similarity was observed with the E-value threshold conservatively set at 1×10^{-3} (1e-3).

No homology was found between the triacylglycerol lipase protein sequence and any known allergenic proteins.

3.3.3 Other allergenic components

Wheat derived material is used in the fermentation medium, and the enzyme preparation contains wheat flour.

3.3.4 Bioinformatic analysis for potential toxicity

The applicant provided results from *in silico* analyses comparing the amino acid sequence for the triacylglycerol lipase protein to known protein toxins identified in the NCBI protein database. Several lipase proteins from toxigenic organisms were identified, which is not unexpected and does not show that the lipase protein expressed in this system is toxigenic to humans. The search did identify some hypothetical proteins and one identified toxin with similarity of sequence greater than 50% however the overlap of sequence was less than 30% and the e-value was greater than 0.1. Due to the low significance of these results, it can be concluded that the toxigenic potential of this protein is low.

3.3.5 Toxicity studies of the enzyme

Reports of two *in vitro* genotoxicity studies with triacylglycerol lipase have been submitted by the applicant: a bacterial reverse mutation test (Ames test) and a chromosomal aberration test in mammalian cells. The applicant also provided a 90 day repeated dose oral toxicity

⁷ www.allergenonline.org

⁸ <http://allergen.nihs.go.jp/ADFS/>

study in rats. All studies were conducted with triacylglycerol lipase, referred to in the study reports as 'Lipase produced with *T. reesei*', Batch number LP 12136B3; RF10625, with a total organic solid (TOS) content of 94.38%.

3.3.5.1 Genotoxicity

Bacterial reverse mutation assay (Sokolowski 2013) Regulatory status: GLP; Conducted according to OECD Test Guideline (TG) 471

The mutagenic potential of lipase produced with *T. reesei* was assessed using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 and TA 102. The assay was performed in triplicate in two independent experiments, both with and without metabolic activation (S9 mix). The first experiment was conducted by the plate incorporation method and the second by the pre-incubation method. The vehicle and solvent control was water. The positive control for all strains in the presence of metabolic activation was 2-aminoanthracene. In the absence of metabolic activation, positive controls were sodium azide (for TA 1535 and TA 100), 4-nitro-o-phenylene-diamine (TA 1537 and TA 98) and methyl methane sulfonate (TA 102). Test item concentrations ranged from 3-5000 µg/plate in experiment I and 33-5000 µg/plate in experiment II, on a TOS basis. To examine a minor increase in the number of revertant colonies in strain TA 98 in the absence of metabolic activation, two further experiments (IIa and IIb) were performed with TA 98 without S9 mix using the pre-incubation method. Experiment IIa used concentrations of 2500 – 10,000 µg/plate. Due to issues with microbial contamination in experiment IIa, this experiment was repeated with and without sterile filtrated test item solution as experiment IIb. Concentrations in experiment IIb without filtration were 2500-10,000 µg/plate, and with filtration concentrations were 2500 and 5000 µg/plate, as concentrations of 7500 and 10,000 µg/plate could not be filtered due to limited solubility.

No precipitation of the test item occurred up to the highest dose, and plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in all strains. No toxicity was observed in the test groups. No significant increase in revertant colony numbers was observed in any strain following treatment with the test item, with or without S9 mix. A minor increase in the number of revertant colonies was observed in experiments I and II in strain TA 98 in the absence of metabolic activation at the highest concentration of the test item, although the threshold of a 2-fold increase over the solvent control was not reached, and the mean value of revertant colonies was in the range of historical control data for the solvent control. In experiments IIa and IIb without filtration, microbial contamination occurred preventing the use of these results. In experiment IIb with filtration, a two-fold increase in the number of revertant colonies was not observed following treatment with the test item. All positive controls produced the expected positive response, demonstrating the validity of the test system used in this study.

It was concluded that lipase produced with *T. reesei* was not mutagenic under the conditions of this study.

Chromosomal aberration test in human lymphocytes (Bohnenberger 2013) Regulatory status: GLP; Conducted according to OECD TG 473

Lipase produced with *T. reesei* was evaluated for its potential to induce structural chromosomal aberrations in human lymphocytes *in vitro*. Two experiments were performed. Experiment I was performed with and without metabolic activation (S9 mix) and the exposure period was 4 hours. In experiment II the exposure period was 22 hours, without S9 mix. Cells were treated 72 hours after mitogenic stimulation with phytohaemagglutinine in all experiments, and chromosomes were prepared for analysis 22 hours after the start of treatment with the test items, and three hours after the addition of the metaphase arresting

substance colcemid. Each concentration was tested in two parallel cultures, and at least 100 metaphases per culture were scored for structural chromosomal aberrations, and 1000 cells per culture were counted for determination of the mitotic index. The highest concentration tested was 5300 µg/mL, corresponding to 5000 µg/mL TOS. Three test concentrations per experiment were used for cytogenetic analysis, based on cytotoxicity observations, the concentrations ranged from 1730.6-5300 µg/mL (approximately 1633-5000 µg/mL TOS) in experiment I without S9, 105.4-322.9 µg/mL (approximately 99-305 µg/mL TOS) in experiment I with S9 and 374.1-1145.8 µg/mL (approximately 353-1081 µg/mL TOS) in experiment II. The vehicle and solvent control was water, and positive controls were ethylmethane sulfonate and cyclophosphamide in the absence and presence of S9, respectively.

No significant increases in chromosomal aberration rates were observed at any of the concentrations of lipase evaluated, with or without metabolic activation. The positive controls produced distinct increases in cells with structural chromosomal aberrations, confirming the validity of the test system.

It was concluded that lipase produced with *T. reesei* did not induce structural chromosomal aberrations in cultured human lymphocytes under the conditions of this study.

3.3.5.2 Short-term studies in animals

*90-day repeated dose oral toxicity study in rats (Braun 2014) Regulatory status: GLP;
Conducted according to OECD TG 408*

Lipase produced with *T. reesei* was administered by oral gavage to Wistar rats (10/sex/group) at doses of 0, 50, 200 and 1000 mg/kg bw/day for a period of 92/93 days. Doses were adjusted to account for the TOS content (94.38%). The vehicle and control was water. Animals were observed for clinical signs before the start of administration and daily thereafter (twice daily on Days 1-3). Detailed behavioural observations were recorded weekly, and a functional observational battery, grip strength and locomotor activity were evaluated in all animals during the final week of the study (Week 13). Food consumption and body weights were recorded weekly. Ophthalmoscopy was performed on all animals during the acclimatisation period, and on animals in the control and high-dose animals during the final week of treatment. Blood samples were collected for analysis after an 18 hour fasting period (with *ad libitum* access to water) on the day of study termination, and urine samples were collected during the fasting period. All animals were killed, necropsied and macroscopic observations were made for all animals. Histopathological observations were performed on organs and tissues from all animals in the control and high-dose groups.

All animals survived until the end of the study and there were no clinical signs of toxicity observed. There were no test-item related findings in the functional observational battery, grip strength, and locomotor activity assessments. No test-item related effects on food consumption, body weight and body weight gain were observed. No ophthalmoscopic changes related to treatment were observed. In haematology, clinical chemistry and urinalysis analyses, no changes of toxicological significance were observed. There were no treatment related changes in absolute or relative organ weights, and no test item related macroscopic or microscopic findings.

Based on the results of this study, it was concluded that the no observed adverse effect level of lipase produced with *T. reesei* was 1000 mg/kg bw/day, the highest dose tested.

3.4 Safety assessment conclusions

There are no public health and safety issues associated with the use of triacylglycerol lipase from *T. reesei* when used as a food processing aid on the basis of the following considerations:

- The production organism *T. reesei* is not pathogenic and is absent in the final enzyme preparation proposed to be used as a food processing aid. Furthermore, *T. reesei* has a history of safe use as the production organism for a number of enzyme processing aids that are already permitted in the Code.
- Molecular characterisation of the production strain has confirmed the sequence of the inserted DNA is as expected and has not undergone any rearrangement, and the introduced DNA is stably inherited.
- Residual enzyme is expected to be present in the final food product but would be inactivated by heat-treatment or non-active because of a lack of substrate.
- Bioinformatic analysis indicated that the enzyme has no biologically relevant homology to known protein allergens or toxins and is unlikely to pose an allergenicity or toxicity concern.
- The enzyme preparation was not mutagenic or clastogenic *in vitro*. The NOAEL in a 90-day repeated dose oral toxicity study in rats was the highest dose tested, 1000 mg/kg bw/day on a TOS basis. This is more than 11,000-fold higher than the applicant's estimate of an individual's theoretical maximal daily intake (0.09 mg TOS/kg bw/day) based on the proposed uses, as stated in the application.

Based on the reviewed toxicological 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 is therefore not required.

The enzyme preparation contains wheat flour.

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