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

2 November 2017
[31-17]

Supporting document 1

Risk and technical assessment report – Application A1130

Triacylglycerol Lipase as a Processing Aid (Enzyme)

Executive summary

Application A1130 seeks approval to use the enzyme triacylglycerol lipase, sourced from *Candida cylindracea*, as a processing aid. Triacylglycerol lipase catalyses the conversion of triglycerides (also called triacylglycerols and lipids, in fats and oils) to diglycerides and mono glycerides. This conversion has flavour-enhancing properties and can also extend the freshness of baked products. The enzyme is intended for use in baking, milk and dairy processing, and fats and oil processing.

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. It was also concluded that the enzyme performs its technological purpose during processing and manufacture of food after which it is inactivated so does not perform any 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 for enzyme preparations used in the production of food.

There are no public health and safety concerns associated with the use of triacylglycerol lipase from *Candida cylindracea* as a food processing aid, on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic. *Candida cylindracea* has a long history of safe use overseas in the production of lipases for a range of industrial purposes, including use as a food processing aid.

- Triacylglycerol lipase was not genotoxic *in vitro*.

- The no observed adverse effect level (NOAEL) in a 13-week repeated dose oral toxicity study in rats was the highest dose tested and corresponds to 10,200 mg/kg bw/day or 581 mg total organic solids (TOS)/kg bw/day. This is more than 5000-fold higher than the Applicant's estimate of an individual's theoretical maximal daily intake (0.102 mg total TOS/kg bw/day) based on the proposed uses in baking, milk and dairy processing, and fats and oil processing, as stated in the Application.

- Triacylglycerol lipase from *Candida cylindracea* does not share amino acid sequence homology with any known allergens. No reports of allergenic concerns resulting from use overseas have been identified.

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 was therefore not required.

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1 Introduction

1.1 Objectives of the assessment

Currently, there are no permissions for the enzyme triacylglycerol lipase derived from *Candida cylindracea* in the *Australia New Zealand Food Standards Code* (the Code). Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

The objectives of this risk and technical assessment 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 concerns that may arise from the use of the triacylglycerol lipase enzyme as a processing aid.

2 Food technology assessment

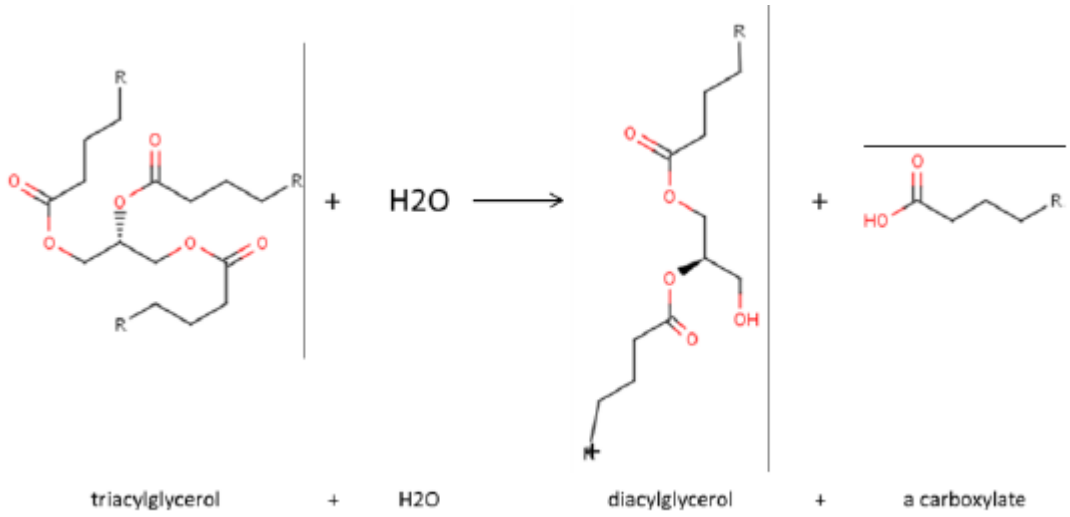
2.1 Characterisation of the enzyme

2.1.1 Identity of the enzyme

Information regarding the identity of the enzyme contained in the Application has been verified using an appropriate enzyme nomenclature reference (IUBMB 2016). Additional information has also been included from this reference.

Generic common name:	Triacylglycerol lipase
Accepted IUBMB[1] name:	Triacylglycerol lipase
Systematic name:	Triacylglycerol acylhydrolase
IUBMB enzyme nomenclature:	EC 3.1.1.3
C.A.S. number:	9001-62-1
Other names:	Lipase; Triglyceride lipase; Tributyrase; Glycerol ester hydrolase; Triglyceride hydrolase; Triglyceride lipase;
Reaction:	triacylglycerol + H_2O = diacylglycerol + a carboxylate

[1] International Union of Biochemistry and Molecular Biology



The enzyme is sourced from a chemically mutated production strain of *C. cylindracea*, which has not been genetically modified.

2.1.2 Technological purpose

Triacylglycerol lipase is used in the manufacture of bakery products and dairy products and in the processing of fats and oils. As noted above triacylglycerol lipase catalyses the conversion of triglycerides (also called triacylglycerols and lipids, in fats and oils) to diglycerides (and mono glycerides) by the selective removal of fatty acids (carboxylates). The lipase catalyses the hydrolysis of short and medium chain fatty acids in preference to long chain fatty acids, selectively from the 1 and 3 positions of the triglyceride.

Triacylglycerol lipase is used to preferentially separate out and convert various fatty acids to produce a variety of structured lipids that provide an enhanced flavour profile.

A common application of lipases (including triacylglycerol lipase) in the dairy industry is to hydrolyse milk fat. The production of short chain fatty acids (such as C4 and C6 fatty acids) which have sharp tangy flavours are used to enhance the flavour of cheese, accelerate cheese ripening, the manufacturing of cheese-like products and the lipolysis of butterfat (Ferreira-Dias et al 2013).

Lipases are also used to extend the freshness of baked products (Bárcenas et al 2003).

The enzyme is inactivated by heating that occurs during the various production steps so it has no technological purpose in the final food products.

2.2 Manufacturing process

2.2.1 Production of the enzyme

The production of the triacylglycerol lipase enzyme preparation occurs by standard enzyme fermentation processes using the source microorganism, *C. cylindracea*. Once the fermentation has been completed, the broth containing the enzyme undergoes a number of separation and concentration steps to produce the final commercial enzyme preparation. The preparation is also standardised to ensure the appropriate concentration of the enzyme in the preparation. The enzyme is sprayed dried onto a solid carrier, being dextrin, so that the enzyme preparation is a powder. The final powdered enzyme preparation contains 7% enzyme and 93% dextrin sourced from cassava.

The production of the enzyme preparation is represented by the schematic in Figure 1 taken from the Application, noting the standardisation occurs after the spray drying step.

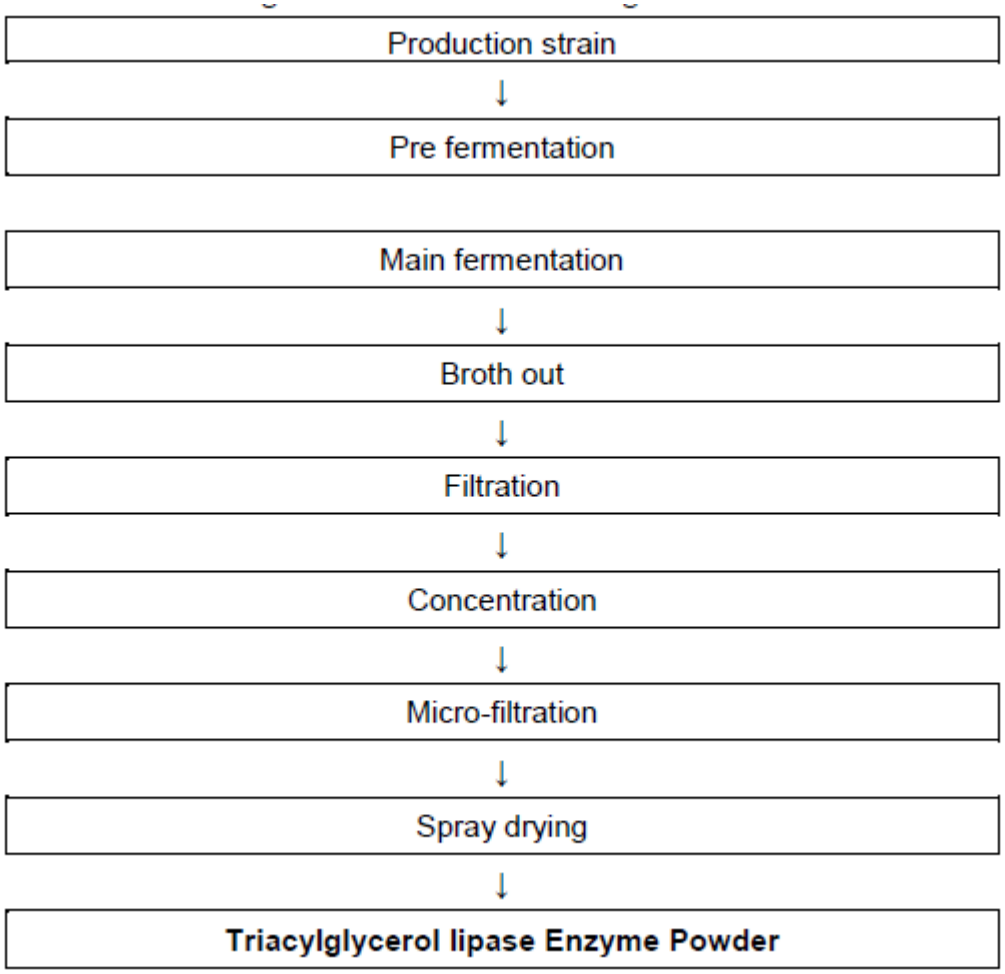


Figure 1: Schematic of the production process of the enzyme preparation

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) in its Compendium of Food Additive Specifications (JECFA 2016) and in the Food Chemicals Codex (Food Chemicals Codex 2014). These primary sources of specifications are listed in the table to section S3—2 of Schedule 3 – Identity and purity. Enzyme preparations need to meet these enzyme specifications. Schedule 3 of the Code also includes specifications for heavy metals (section S3—4) if they are not specified within specifications in sections S3—2 and S3—3.

Table 2 provides a comparison of the product specifications with the international specifications established by JECFA as well as those detailed in the Code (as applicable).

Table 1: Specifications for commercial enzyme preparation compared to JECFA and the Code specifications for enzymes

Analysis	Specifications		
	Amano Product	JECFA	Australia New Zealand Food Standards Code (metals) (section S3—4)
Lead (mg/kg)	<0.10	≤ 5	≤2
Arsenic (mg/kg)	<1.0	-	≤1
Mercury (mg/kg)	<0.001	-	≤1
Cadmium (mg/kg)	<0.001	-	≤1
Antimicrobial activity	Not detected	Not detected	-
Coliforms (cfu/g)	≤10	≤30	-
Salmonella (in 25 g)	Absent	Absent	-
E. coli	Absent/10 g	Absent/25 g	-

Certificate of Analyses (CoA) were provided on three samples of the enzyme preparation which indicated compliance with the specifications. Based on the CoA against the above specifications, the final enzyme preparation meets international and Code specifications for enzyme preparations used in the production of food.

2.2.3 Stability

Triacylglycerol lipase has optimal activity between pH 5 to 10, with the peak at pH 7. Its optimum activity is achieved around 50°C, within the range of 20–60°C. The enzyme is inactivated at 70°C. The enzyme activity is stable at pH 7 between the temperature range of 40–50°C.

The powdered enzyme preparation stored in an airtight bag was assessed to be stable up to 12 months when stored at 25°C, and 3 months when stored at 40° C.

2.3 Food technology conclusion

FSANZ concludes that the stated purpose of this enzyme preparation; namely, for use as a processing aid intended for use in baking, milk and dairy processing, and fats and oil processing is clearly articulated in the Application. Triacylglycerol lipase is used to convert fats and oils in various primary food materials to produce different fatty acids which provide enhanced flavour properties. 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. That is, it performs its technological purpose during processing and manufacture of food after which it is inactivated so does not perform any 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 Hazard assessment

3.1 Background

3.1.1 Scope of the hazard assessment

The aims of the current hazard assessment are to:

- Review the available data on the toxicology of triacylglycerol lipase from *C. cylindracea* to determine its safety as a food processing aid

- If appropriate, establish a health-based guidance value.

3.2 Hazard of the production organism

C. cylindracea has been used for a long time in the biomedical, cosmetic, detergent and food industries, as a lipase producer.

C. cylindracea is not known to be pathogenic or toxigenic, and a search of the available scientific literature did not identify any animal studies or clinical reports that suggest a potential safety concern. The Ogataea clade to which it belongs does not include pathogenic yeasts associated with human infections (EFSA 2014; EFSA 2017). As *C. cylindracea* is a yeast it does not produce mycotoxins.

The European Food Safety Authority (EFSA) has recommended qualified presumption of safety (QPS) status for *C. cylindracea* when used for the production of enzymes (EFSA 2014; EFSA 2017).

3.3 Hazard of the enzyme

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

There is evidence that triacylglycerol lipase from *C. cylindracea* has been used in Japan for many years in food processing (Arnold et al 1975) and it is also permitted as a food additive that may include its use as a food enzyme in China.

3.4 Evaluation of toxicity studies of the enzyme product

3.4.1 Genotoxicity

Two genotoxicity studies were submitted by the Applicant, a bacterial reverse mutation assay and a chromosomal aberration assay in mammalian cells. The test article, lipase liquid concentrate, is a material obtained after concentration from normal commercial production. This concentrate does not include the spray auxiliary agents (the diluent or the stabiliser).

A bacterial reverse mutation assay of lipase conc (Candida cylindracea) – Oguma (Study Director); Bozo Research Center study number T-1056 (2012)

The study was conducted in compliance with the Ministry of Health and Welfare of Japan (JMHW) Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs, as well as the JMHW Guidelines on Genotoxicity Testing of Pharmaceuticals. The test material was a liquid lipase concentrate with an enzyme activity level of 71,000 μ/mL. Water was used as the vehicle.

Tester strains were the *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, plus *Escherichia coli* WP2 uvrA. Based on the findings of a dose range finding study five test article concentrations were used in the main study: 100, 50, 25, 12.5 and 6.25 % solution (0.1 mL/plate). The test was conducted by the preincubation method with and without metabolic activation. The criteria for a positive response were a two-fold increase in the reversion rate over that of the negative control, and demonstration of a dose-response.

All positive controls gave the expected mutagenic response, while no evidence of

mutagenicity was observed with the test substance. It was concluded that the test article had no mutagenic activity under the conditions of the study.

Chromosome aberration test of lipase concentrate (Candida cylindracea) in cultured mammalian cells – Fujiwara (Study Director); Bozo Research Center study number T-G050 (2012)

The study was conducted in compliance with the JMHW Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs, as well as the JMHW Guidelines on Genotoxicity Testing of Pharmaceuticals. The test material was a liquid lipase concentrate with an enzyme activity level of 71,000 μ/mL.

The test material was examined for its potential to induce structural chromosome aberrations in Chinese hamster lung fibroblast (CHL/IU) cells in both the absence and presence of S9 fraction. The solvent and negative control was water. The positive control substances were mitomycin C in the absence of S9 fraction and cyclophosphamide in the presence of S9 fraction. Both a short-term treatment assay and a continuous exposure assay were conducted. The continuous exposure assay was conducted only in the absence of metabolic activation. The exposure period in the short-term treatment assay was 6 hours, and cells were harvested 18 hours following removal of the test article. In the continuous exposure assays colcemid® was added approximately 2 hours before the end of the 24 or 48 hour culture period. All assays were conducted in duplicate.

Based on the results of a cell-growth inhibition test the dose concentrations selected for the short-term treatment assays with or without metabolic activation were 12.5, 25, 50 and 100% of test article (0.5 mL/plate). For the continuous exposure assay, test article dose concentrations of 0.391, 0.781, 1.56 and 3.13% were used when the exposure period was 24 hours, and concentrations of 0.0977, 0.195, 0.391 and 0.781% were used for the 48 hour exposure. The highest concentrations in the continuous exposure tests resulted in cytotoxicity of greater than 50% compared with negative controls.

A < 5% incidence of cells with structural and numerical chromosome aberrations was considered to indicate a negative response, an incidence of ≤ 5 to < 10% an equivocal response and an incidence of ≥ 10% was considered a positive response.

In both the short-term and continuous treatment tests, the incidence of cells with chromosome aberrations excluding gaps (an index for structural chromosome aberrations), and the incidence of polyploid cells, were in the range considered negative at all dose concentrations with and without metabolic activation. There was also no indication of an increased incidence of chromosome aberrations compared with the negative controls. Marked increases in the number of cells with structural chromosome aberrations were seen in the positive control groups, confirming the validity of the study.

It was concluded that the test substance did not induce structural chromosome aberrations under the conditions of this study.

3.4.2 Animal studies

A 13-week oral toxicity study of lipase concentrate (*Candida cylindracea*) in rats – Yamamoto (Study Director); Bozo Research Center study number B-7279 (2013)

The study was conducted in compliance with the JMHW Ordinance on Standard for Conduct of Non-Clinical Studies on Safety of Drugs, as well as JMHW guidelines for repeated-dose toxicity studies, food additives and toxicity studies for pharmaceuticals.

The test material was the liquid lipase concentrate with an enzyme activity level of 71,000 µ/mL. The test vehicle and negative control was water. Lipase concentrate was administered daily by oral gavage to Sprague Dawley Crl:CD(SD) SPF rats (12/sex/group) for 13 weeks. Rats were acclimatised for 8 days prior to the first day of treatment, at which time they were approximately 6 weeks old. Animals were housed individually with *ad libitum* access to food and water. Animal room conditions were standard laboratory husbandry conditions. Rats were gavaged with a dose volume of 10 mL/kg bw/day. Based on the findings of a prior 2-week oral gavage study in rats, concentrations of the test substance administered were 25, 50 or 100% lipase concentrate, with a vehicle control group also included. The theoretical lipase activity of these test concentrations was 17,750, 35,550 and 71,000 µ/mL, respectively. Measured activities were 103–107% of the theoretical activities in the first week of the study and 96–110 % in Week 13.

Survival, clinical signs, body weight and food consumption were assessed throughout the course of the study, and an ophthalmological examination was performed before the start of the administration period and again in Week 13 of administration. Urine samples were collected in Week 13 and blood samples were collected at scheduled necropsy. Animals were killed by exsanguination under anaesthesia. A complete gross necropsy was conducted on all rats and organ weights were determined for brain, thyroid, adrenals, thymus, spleen, heart, lung, salivary gland, liver and kidney as well as the testis, prostate and seminal vesicle of males and ovary and uterus of females. Tissues were preserved and processed for histopathological examination.

No deaths occurred in any group, and no treatment-related clinical signs were observed. No treatment-related effects on body weight or food consumption were seen. A statistically significant reduction in body weight was seen in males in the 50% dose group from Day 28 as well as reduced food consumption on Days 28–35 and Day 73, but these were considered to be incidental findings as a dose-response was not observed. There were no treatment-related changes in organ weight, ophthalmology, urinalysis, haematology, blood chemistry, necropsy or histopathology.

The highest dose of lipase concentrate used in this study, 10 mL/kg bw/day of a 100% solution, was identified as the No Observed Adverse Effect Level (NOAEL). The Application states that the NOAEL corresponds to 10,200 mg/kg bw/day of the test substance. Based on a total organic solid (TOS) content of 5.7% this equates to 581 mg TOS/kg bw/day.

3.4.3 Bioinformatic analysis for potential allergenicity

An *in silico* analysis was used to compare the amino acid sequence of triacylglycerol lipase with that of known allergens using the Allergen Database for Food Safety. Searches were conducted to investigate whether there are matches for:

- more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty, or
- identity of 8 contiguous amino acids.

A further 80 amino acid alignment comparison was conducted using the Allergen Online Database. These homology assessments are consistent with the recommendations of international organisations for screening of new food enzymes or newly expressed proteins in genetically modified plants for potential allergenicity (FAO/WHO 2001; WHO 2016).

The searches did not identify any matches with known allergens.

History of use

Triacylglycerol lipase from *C. cylindracea* has been used in a variety of industries including the biomedical, cosmetic, detergent and food industries for many years (Hasan el al. 2006; Arnold et al. 1975; Pandey et al. 1999; EFSA 2014; EFSA 2017). No reports of safety concerns including allergenicity have been identified, suggesting a history of safe use.

Overall, it is concluded that triacylglycerol lipase does not have the characteristics of a potential food allergen and ingestion of any residual triacylglycerol lipase in food products is unlikely to pose an allergenicity concern.

Other components

Soybean oil is used in the fermentation media and is a potential food allergen. However, refined soybean oil is used and, as concluded in the risk assessment for the FSANZ proposal on exemptions from allergen labelling (P1031), fully refined soybean oil presents negligible risk to soybean allergic consumers. In addition, analysis of triacylglycerol lipase indicates that soybean protein is not detected above the limit of detection of 1 mg/kg, and the enzyme is added at very low levels to foods (maximum 0.082%). Therefore exposure to any potential residual soy allergens in final food products will be very low and is not likely to be of allergenic concern.

3.5 Risk assessment discussion and conclusions

There are no public health and safety concerns associated with the use of triacylglycerol lipase from *C. cylindracea* as a food processing aid, on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic. *C. cylindracea* has a long history of safe use overseas in the production of lipases for a range of industrial purposes, including use as a food processing aid.

- Triacylglycerol lipase was not genotoxic *in vitro*.

- The enzyme preparation caused no observable adverse effects. The no observed adverse effect level (NOAEL) in a 13-week repeated dose oral toxicity study in rats was the highest dose tested and corresponds to 10,200 mg/kg bw/day or 581 mg TOS/kg bw/day. This is more than 5000-fold higher than the Applicant's estimate of an individual's theoretical maximal daily intake (0.102 mg TOS/kg bw/day) based on the proposed uses in baking, milk and dairy processing, and fats and oil processing, as stated in the Application.

- Triacylglycerol lipase from *C. cylindracea* does not share amino acid sequence homology with any known allergens. No reports of allergenic concerns resulting from use overseas have been identified.

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 was therefore not required.

4 Conclusion

This risk and technical assessment considered the technological suitability, potential hazard of the enzyme and its source microorganism including potential allergenicity.

It was concluded that the proposed use of the enzyme is technologically justified in the form and prescribed amounts as a processing aid and has been demonstrated to be effective. The evidence presented is sufficient to determine that triacylglycerol lipase derived from *C. cylindracea* is unlikely to pose a public health and safety concern when used as a food processing aid.

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