SCIENTIFIC OPINION

efsa JOURNAL

ADOPTED: 8 June 2023

doi: 10.2903/j.efsa.2023.8099

Safety evaluation of the food enzyme triacylglycerol lipase from the non-genetically modified *Rhizopus arrhizus* strain AE-TL(B)

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Abstract

The food enzyme triacylglycerol lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) is produced with the non-genetically modified *Rhizopus arrhizu*s strain AE-TL(B) by Amano Enzyme Inc. The food enzyme was considered free from viable cells of the production organism. It is intended to be used in the modification of fats and oils by interesterification and in the manufacture of enzyme-modified dairy ingredients. Dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 0.057 mg TOS/kg body weight (bw) per day in European populations. Genotoxicity tests did not indicate a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 1,960 mg TOS/kg bw per day, the highest dose tested, which, when compared with the estimated dietary exposure, resulted in a margin of exposure of at least 34,386. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no match found. The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure cannot be excluded, but the likelihood is low. Based on the data provided, the Panel concluded that this food enzyme does not give rise to safety concerns, under the intended conditions of use.

Keywords: food enzyme, triacylglycerol lipase, triacylglycerol acylhydrolase, EC 3.1.1.3, *Rhizopus arrhizu*s, non-genetically modified microorganism

Requestor: European Commission

Question number: EFSA-Q-2014-00112

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Legal notice: The full opinion will be published in accordance with Article 12 of Regulation (EC) No 1331/2008 once the decision on confidentiality will be received from the European Commission.

Acknowledgements: The Panel wishes to thank the following for the support provided to this scientific output: Magdalena Andryskiewicz, Ana Gomes, Simone Lunardi and Kim Rygaard Nielsen†.

Declaration of interest: If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

Suggested citation: EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Lambré C, Barat Baviera JM, Bolognesi C, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mengelers M, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Glandorf B, Herman L, Toldrá F, Aguilera J, Kovalkovičová N, Liu Y, Maia J, di Piazza G and Chesson A, 2023. Scientific Opinion on the safety evaluation of the food enzyme triacylglycerol lipase from the non-genetically modified *Rhizopus arrhizus* strain AE-TL(B). EFSA Journal 2023;21(8):8099, 17 pp. https://doi.org/10.2903/j.efsa.2023.8099

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

† Deceased.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.

'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008[1] on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

- it does not pose a safety concern to the health of the consumer at the level of use proposed;
- there is a reasonable technological need;
- its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) lays down the administrative, technical and toxicological data required.

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7(2) of Regulation (EC) No 1332/2008[1] on food enzymes.

Three applications have been introduced by the applicant Amano Enzyme Inc. for the authorisation of the food enzymes triacylglycerol lipase from *Rhizopus oryzae* (strain AE-TL), triacylglycerol lipase from *Candida cylindracea* (strain AE-LAYH), and leucyl aminopeptidase from *Aspergillus oryzae* (strain AE-MB).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

1.1.2. Terms of Reference

The European Commission (EC) requests the European Food Safety Authority (EFSA) to carry out the safety assessments on the following food enzymes triacylglycerol lipase from *Rhizopus oryzae* (strain AE-TL), triacylglycerol lipase from *Candida cylindracea* (strain AE-LAYH), and leucyl

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, pp. 15–24.

aminopeptidase from *Aspergillus oryzae* (strain AE-MB) in accordance with Article 17.3 of Regulation (EC) No 1332/2008[1] on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme triacylglycerol lipase from *Rhizopus oryzae* (strain AE-TL). The code AE-TL used in this application covers multiple strains. Following a clarification in July 2022,[4] the applicant specified the production strain as AE-TL(B). The applicant confirmed that this production strain is deposited as [redacted] and all provided data, analyses and studies were performed with the food enzyme produced with this production strain AE-TL(B).

Recent data identified the production microorganism as *Rhizopus arrhizus* (Section 3.1).[5] Therefore, this name *R. arrhizus* strain AE-TL(B) will be used in this opinion instead of *R. oryzae* (strain AE-TL).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme triacylglycerol lipase from *R. arrhizus* strain AE-TL(B) (formerly *R. oryzae* strain AE-TL).

Additional information was requested from the applicant during the assessment process on 7 April 2014, 20 February 2015, 6 July 2020, 12 April 2022 and 10 October 2022 and received on 10 July 2014, 14 April 2015, 4 June 2021, 8 July 2022 and 22 December 2022 (see 'Documentation provided to EFSA').

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009) and following the relevant guidance documents of the EFSA Scientific Committee.

The 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA CEF Panel, 2009) as well as the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) have been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance with the updated 'Scientific Guidance for the submission of dossiers on food enzymes' (EFSA CEP Panel, 2021a).

3. Assessment[6]

IUBMB nomenclature	Triacylglycerol lipase
Systematic name	Triacylglycerol acylhydrolase
Synonyms	Lipase; triglyceride lipase
IUBMB No	EC 3.1.1.3
CAS No	9001-62-1
EINECS No	232-619-9

Triacylglycerol lipases catalyse, in the presence of water, the hydrolysis of the ester linkages in triacylglycerols, resulting in the generation of glycerol, free fatty acids, diacylglycerols and monoacylglycerols. In the absence of water, or at a very low concentration of water, interesterification, i.e. the exchange of free fatty acids in positions 1 and 3 between two or more triacylglycerols, may occur. The food enzyme under assessment is intended to be used in the modification of fats and oils by interesterification and in the manufacture of enzyme modified dairy ingredients.[7]

[4] Technical dossier/Additional information, 8 July 2022/Answer to point 1.
[5] Technical dossier/Additional information, 4 June 2021.
[6] Technical dossier/p. 4–5, 24, 28, 52.
[7] Technical dossier/Additional information, 4 June 2021; Technical dossier/Additional information, 22 December 2022.

3.1. Source of the food enzyme[8]

The triacylglycerol lipase is produced with the non-genetically modified filamentous fungus *R. arrhizus* strain AE-TL(B),[9] which is deposited at [redacted] with the deposit number [redacted].[10]

The production strain was identified as *R. arrhizus* [redacted].[11] *R. oryzae* is currently reclassified into *R. arrhizus* (Walther et al., 2013; Dolatabadi et al., 2014).

The production strain was derived from *Rhizopus oryzae* strain 4697 by conventional mutagenesis.

3.2. Production of the food enzyme[12]

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[13], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.

The production strain is [redacted]. The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.

The food enzyme may be used in an immobilised form.[14] It is [redacted].[15] No cross-linking agents are used in the preparation of the immobilised enzyme.[15]

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The triacylglycerol lipase is a single polypeptide chain of [redacted] amino acids.[16] The molecular mass, derived from the amino acid sequence, was calculated to be [redacted] kDa.[17] The consistency of the protein profile of the food enzyme was illustrated by size exclusion chromatography of three batches.[18] No other enzyme activities were reported.[19]

The hydrolytic activity of the triacylglycerol lipase[20] is quantified based on the measurement of the amount of free fatty acids released from an olive oil emulsion in the presence of sodium taurocholate over a 10 min interval (37°C) and is expressed in Fungi-Lipase International Units/g (FIP Units/g) and quantified relative to an enzyme standard.

[8] Technical dossier/p. 9, 31; Technical dossier/Additional information, 4 June 2021/Annex 1; Technical dossier/Additional information, 8 July 2022.
[9] Technical dossier/Additional information, 8 July 2022.
[10] Technical dossier/Additional information, 4 June 2021/Annex 1.
[11] Technical dossier/Additional information, 4 June 2021/Annex 2.
[12] Technical dossier/p. 10, 34–38; Technical dossier/Annex 4, Annex 5, Annex 6; Technical dossier/Additional information, 10 July 2014/p. 6/Annex 1; Technical dossier/Additional information, 14 April 2015/Answer to point 1.
[13] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.
[14] Technical dossier/Additional information, 14 April 2015/p. 5; Technical dossier/Additional information, 4 June 2021/Answer to Point 4; Technical dossier/Additional information, 22 December 2022/Answer to Point 1.
[15] Technical dossier/Additional information, 22 December 2022/Answer to Point 1.
[16] Technical dossier/p. 27; Technical dossier/Additional information, 4 June 2021/Answer to Point 3.
[17] Technical dossier/p. 28; Technical dossier/Additional information, 4 June 2021/Answer to Point 3.
[18] Technical dossier/p. 25–26.
[19] Technical dossier/p. 26.
[20] Technical dossier/p. 29; Technical dossier/Annex 2.

The trans-esterification activity is determined based on the formation of methyl caprate from tricaprylin measured by gas chromatography. One Trans-esterification Unit (TEU) is defined as the quantity of enzyme required to form 1 nmoL of methyl caprate per minute.[21]

The triacylglycerol lipase is active at temperatures up to 70°C, with an optimum of 35–40°C (pH 7) and within a pH range of 4 to 9 with an optimum of pH 6–7 (37°C). The thermostability was tested by pre-incubation at different temperatures for 30 min. The enzyme activity was stable up to 50°C. With the increasing temperature activity was reduced, with no activity detected above 70°C.[22]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme preparation were provided for three batches used for commercialisation (batches 1–3) and three batches (batches 4–6) produced for the toxicological tests (Table 1).[23] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 29.1% and the mean enzyme hydrolysis activity/TOS ratio was 8,080 FIP Units/mg TOS. The mean trans-esterification activity/TOS ratio of the three batches used for commercialisation was 36.1 TEU/mg TOS.[24]

Table 1: Compositional data of the food enzyme preparation[7]

Parameters	Unit	Batches					
		1	2	3	4[(a)24]	5[(b)]	6[(c)]
Triacylglycerol lipase activity (hydrolysis)	FIP Units/g[(d)]	2,610,000	1,810,000	2,490,000	2,784,000	2,660,000	3,170,000
Triacylglycerol lipase activity (trans-esterification)	TEU/g[(e)]	7,800	18,000	6,600	NA[(g)]	NA[(g)]	NA[(g)]
Protein	%	26.2	32.3	28.7	76.3	79.4	93.8
Ash	%	3.0	2.1	2.2	0.7	0.7	0.8
Water	%	5.4	5.9	6.0	3.7	2.0	1.2
[redacted]	%	65.4	59.7	63.1	0	0	0
Total organic solids (TOS)[(f)]	%	26.2	32.3	28.7	95.7	97.3	98.0
Hydrolysis activity/TOS	FIP Units/mg TOS	9,961.8	5,603.7	8,675.9	2,909	2,734	3,235
Trans-esterification activity/TOS	TEU/mg TOS	29.8	55.7	22.9	NA[(g)]	NA[(g)]	NA[(g)]

(a): Batch used for Ames test.
(b): Batch used for *in vitro* chromosomal aberration test.
(c): Batch used for a repeated dose 90-day oral toxicity study in rats.
(d): FIP Units: Fungi-Lipase International Units (see Section 3.3.1).
(e): TEU: Trans-Esterification Units (see Section 3.3.1).
(f): TOS calculated as 100% - % water - % ash - % diluent.
(g): NA: not analysed.

3.3.3. Purity

The lead content in the three commercial batches and in the three batches used for toxicological studies was below 5 mg/kg,[25] which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). In addition, arsenic,

[21] Technical dossier/Additional information, 10 July 2014/p. 7/Annex 2.
[22] Technical dossier/p. 29–30; Technical dossier/Annex 2; Technical dossier/Additional information, 14 April 2015/Answer to Point 2.
[23] Technical dossier/p. 25, 46; Technical dossier/Annex 1, Annex 2, Annex 10.1/p. 37; Technical dossier/Additional information, 10 July 2014/Answer to Point 2 and 3; Technical dossier/Additional information, 4 June 2021/Answer to Point 2; Technical dossier/Additional information, 22 December 2022.
[24] Technical dossier/Additional information, 4 June 2021/Answer to Point 2.
[25] Technical dossier/p. 9, 27, 46, 53; Technical dossier/Annex 1, Annex 3; Technical dossier/Additional information, 10 July 2014/Answer to Point 2 and 3.

cadmium and mercury contents were below the limits of quantification (LoQ) of the employed methods.[26,27]

The food enzyme preparation complies with the microbiological criteria for total coliforms, *Escherichia coli* and *Salmonella*, as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).[28] In addition, the applicant provided the results of the determination of *Clostridium perfringens* and coagulase positive *Staphylococcus* in the three commercial batches, which were of no concern.[29] No antimicrobial activity was detected in any of the tested batches.[30]

Strains of *Rhizopus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites. The presence of total aflatoxins, aflatoxin B1, B2, G1, G2, deoxynivalenol, HT-2 toxin, T-2 toxin, zearalenone, ochratoxin A and sterigmatocystin was examined in the three food enzyme preparation batches. All were below the limits of quantification (LoQ) of the applied analytical methods.[31,32] Adverse effects caused by the possible presence of other secondary metabolites were addressed by the toxicological examination of the food enzyme TOS.

The Panel considered that the information provided on the purity of the food enzyme was sufficient.

3.3.4. Viable cells of the production strain[33]

The absence of viable cells of the production strain in the food enzyme was demonstrated in three independent batches analysed in triplicate. [redacted]. No colonies of the production strain were detected.

3.4. Toxicological data[34]

A battery of toxicological tests, including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test and a repeated dose 90-day oral toxicity study in rats, was provided. The batches 4, 5 and 6 (Table 1) used in these studies[35] were obtained by freeze drying of the concentrated food enzyme solutions, without the addition of [redacted]. Table 1 shows that the food enzyme batches used for the toxicological assays had a lower hydrolysis activity/mg TOS values compared with the three food enzyme batches used for commercialisation and were considered suitable as test items.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 and 472 (OECD, 1995a,b) and Japanese Guidelines (Notification No. 261 of Ministry of Labor, Japan, 1985 and Notification No. 24 of Japanese Ministry of Health and Welfare (JMHW), 1989) and following Japanese GLP.[36] Four strains of *Salmonella* Typhimurium (TA1535, TA100, TA1537, TA98) and *Escherichia coli* WP2*uvrA* were used with or without metabolic activation (S9-mix), applying the pre-incubation method.

[26] Technical dossier/Annex 1, Annex 3; Technical dossier/Additional information, 14 April 2015.
[27] Technical dossier/Annex 1: Limit of quantification (LoQ): Pb = 0.005 mg/kg; As = 0.002 mg/kg; Cd = 0.001 mg/kg; Hg = 0.001 mg/kg.
[28] Technical dossier/p. 9, 27, 46, 53; Technical dossier/Annex 1, Annex 3; Technical dossier/Additional information, 14 April 2015.
[29] Technical dossier/Annex 3, Annex 1; Technical dossier/Additional information, 14 April 2015/Answer to point 6: Limit of quantification (LoQ) = 10 cfu/g.
[30] Technical dossier/p. 9, 27, 46, 53; Technical dossier/Annex 1, Annex 3; Technical dossier/Additional information, 10 July 2014.
[31] Technical dossier/p. 9, 27, 46, 53; Technical dossier/Annex 1, Annex 3; Technical dossier/Additional information, 14 April 2015/ Answer to point 6: Limit of quantification (LoQ): aflatoxin B1 = 0.2 μg/kg; aflatoxin B2 = 0.2 μg/kg; aflatoxin G1 = 0.2 μg/kg; aflatoxin G2 = 0.2 μg/kg; total aflatoxin = 0.8 μg/kg; deoxynivalenol (vomitoxin) = 20 μg/kg; HT-2 toxin = 10 μg/kg; T-2 toxin = 10 μg/kg; zearalenone = 10 μg/kg; ochratoxin A = 0.5 μg/kg; sterigmatocystin = 10 μg/kg.
[32] Technical dossier/Additional information, 14 April 2015.
[33] Technical dossier/Additional information, 4 June 2021/Annex 3.
[34] Technical dossier/p. 12–13, 16–17, 44–47; Technical dossier/Additional information, 10 July 2014/Answer to Point 2 and 3.
[35] Technical dossier/Additional information, 10 July 2014/Answer to Point 2 and 3.
[36] Technical dossier/p. 44–45; Technical dossier/Annex 8.

Two separate experiments were carried out in triplicate, using five different concentrations of the food enzyme, ranging from 313 to 5,000 μg/plate, corresponding to 299.5, 598, 1,196, 2,393 and 4,785 μg TOS/plate.

No cytotoxicity was observed at any concentration of the test substance. Upon treatment with the food enzyme, there was no biologically relevant increase in the number of revertant colonies above the control values, in any strain tested, with or without S9-mix.

The Panel concluded that the food enzyme triacylglycerol lipase did not induce gene mutations under the test conditions applied in this study.

3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* mammalian chromosomal aberration test was carried out according to OECD Test Guideline 473 (OECD, 1983), Japanese Guideline (Notification No. 24 of JMHW, 1989) and following Japanese GLP.[37]

Two separate experiments were performed in duplicate cultures of Chinese hamster lung cells (CHL/IU). The cell cultures were treated with the food enzyme either with or without metabolic activation (S9-mix). The highest concentrations applied were selected on the basis of the results of a range finding study. In the first experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations of 280, 400 and 570 μg/mL (corresponding to 272, 389 and 555 μg TOS/mL) in a long-term treatment (24 h exposure without recovery period) and at concentrations of 200, 280 and 400 μg/mL (corresponding to 195, 272 and 389 μg TOS/mL) in a long-term treatment (48 h exposure without recovery period) without S9-mix. In the second experiment, cells were exposed to the food enzyme and scored for chromosomal aberrations at concentrations of 1,300, 1,800 and 2,500 μg/mL (corresponding to 1,265, 1,751 and 2,433 μg TOS/mL) in a short-term treatment (6 h exposure and 18 h recovery period) without S9-mix, and at concentrations of 6.3, 13 and 25 μg/mL (corresponding to 6.13, 12.6 and 24.3 μg TOS/mL) with S9-mix.

In the long-term treatment, cytotoxicity higher than 50% was observed at the highest concentrations tested in the 24 and 48 h treatments. The frequency of structural and numerical chromosomal aberrations was not statistically significantly different to the negative controls at any concentrations tested.

The Panel concluded that the food enzyme triacylglycerol lipase did not induce an increase in the frequency of structural and numerical chromosomal aberrations under the test conditions applied in this study.

3.4.2. Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study was performed following GLP and in accordance with the guidelines of Japanese Ministry of Health and Welfare (Ordinance No. 21 and No. 424 of JMHW, 1997a,b).[38] The study is in accordance with OECD Test Guideline 408 (OECD, 1998), but no functional observations were performed. The Panel considered that this deviation does not impact on the evaluation of the study.

Groups of 12 male and 12 female Sprague–Dawley SPF (Crj:CD(SD)IGS) rats received by gavage the food enzyme in doses of 500, 1,000 and 2,000 mg/kg body weight (bw) per day (corresponding to 490, 980 and 1,960 mg TOS/kg bw per day) for 90 days. Controls received the vehicle (water for injection). A recovery control and a high-dose group were included in the study, each comprising six males and six females and terminated 4 weeks after the end of treatment.

No mortality was observed.

Haematological investigation revealed a statistically significant decrease in a differential count of segmented neutrophils in mid-dose males (−34%) at the end of dosing, a statistically significant increase in a differential count of lymphocytes (+10%) and a decrease in a differential count of segmented neutrophils (−31%) in high-dose females at the end of the recovery period. The Panel considered the changes as not toxicologically relevant as there were no changes in other relevant parameters (changes in total leukocyte count), there was no dose–response relationship (segmented neutrophils at the end of dosing), the changes were small (lymphocytes) and were not present at the end of dosing.

[37] Technical dossier/p. 45; Technical dossier/Annex 9.
[38] Technical dossier/p. 45–46; Technical dossier/Annex 10.1, Annex 10.2, Annex 10.3.

Clinical chemistry investigations revealed at the end of dosing a statistically significant decrease in aspartate aminotransferase (AST) activity (−22%) and an increase in total triglyceride concentration (+71%) in low-dose males, and an increase in alanine aminotransferase (ALT) activity (+14%) in high-dose males. At the end of the recovery period, a statistically significant decrease in blood urea nitrogen (BUN) (−7%) and an increase in albumin to globulin (A/G) ratio (+7%) in high-dose males, and a decrease in glucose level (−15%) in high-dose females were reported. The Panel considered the changes as not toxicologically relevant as they were only observed in one sex (all parameters), the changes were small (AST and ALT at the end of dosing, BUN and A/G ratio at the end of the recovery period), there was no dose–response relationship (AST and triglycerides at the end of treatment), there were no changes in other relevant parameters (for ALT no changes in other liver enzymes; for A/G ratio in total protein or albumin), the changes were not present at the end of dosing (all changed parameters at the end of the recovery period).

Urinalysis, in week 5 of dosing, revealed a statistically significant increase in proteins and ketones in high-dose males, and a decrease in pH and proteins, and an increase in urine volume (+40%) accompanied by an increase in water intake (+215%) in mid-dose females. In week 13 of dosing, a statistically significant decrease in urinary pH in high-dose males and in low- and high-dose females, an increase in phosphate crystals in sediment in high-dose males and a decrease in sodium concentration (−24%) in low-dose females were observed. The Panel considered the changes as not toxicologically relevant as they were transient (all parameters from week 5), there was no dose–response relationship (all parameters in mid-dose females in week 5, sodium), the changes were only observed in one sex (ketones, phosphate crystals, urine volume), there was no consistency between the changes in males and females (protein in week 5) and there were no histopathological changes in the kidneys or changes in electrolytes in the blood. At the end of recovery period, a statistically significant decrease in urinary potassium concentration (−23%) in high-dose females was reported. The Panel considered this change as not toxicologically relevant as it was not observed at the end of the dosing period.

Statistically significant changes in organ weights at the end of dosing included a decrease in the relative heart weight (−7%) in high-dose males and of the lungs in low- and high-dose males (−6% and −6%, respectively), and an increase in the relative liver weight in low- and mid-dose males (+11 and +8%, respectively). The Panel considered the changes as not toxicologically relevant as they were only observed in one sex, there was no dose–response relationship (lungs, liver), the changes were small (heart, liver) and there were no macroscopic or histopathological changes in the organs.

After the recovery period, there was a statistically significant decrease in the relative salivary gland weight (−9%) and in the relative adrenal weight (−18%) in high-dose males, and an increase in the absolute and relative ovary weights (+18% and +22%, respectively) in high-dose females. The Panel considered the changes as not toxicologically relevant as they were not observed at the end of the dosing period and there were no histopathological changes in salivary glands, adrenals and ovaries.

No other statistically significant or biologically relevant differences to controls were reported.

The Panel identified a no observed adverse effect level (NOAEL) of 1,960 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

The allergenicity assessment considered only the food enzyme and not any carrier or other excipients that may be used in the final formulation.

The potential allergenicity of triacylglycerol lipase produced with non-genetically modified *R. arrhizus* strain AE-TL(B) was assessed by comparing its amino acid sequence with those of known allergens according to the 'Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (EFSA GMO Panel, 2010). Using a higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.[39]

No information is available on oral and respiratory sensitisation or elicitation reactions of this triacylglycerol lipase.

Respiratory allergy following occupational inhalation of triacylglycerol lipase has been reported (Elms et al., 2003; Martel et al., 2010). Brant et al. (2004) reported occupational asthma in two patients in the detergent industry, caused by cellulase and lipase from *Aspergillus oryzae*. In addition,

[39] Technical dossier/p. 13, 47–48; Technical dossier/Annex 6; Technical dossier/Additional information, 4 June 2021/Annex 4.

there were case reports of allergies due to inhalation of a digestive enzyme drug containing α-amylase and lipase derived from porcine pancreas (Shin et al., 2008). However, some studies have shown that adults with occupational asthma caused by an enzyme used in food can commonly ingest the corresponding respiratory allergens without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Brisman, 2002; Poulsen, 2004; Armentia et al., 2009). Information on adverse reactions upon ingestion of triacylglycerol lipase in individuals sensitised through the respiratory route has not been reported.

[redacted][15,40] that may cause allergies or intolerances (listed in the Regulation (EU) No 1169/2011[41]) are used as raw materials. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the fungal biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel considered that no potentially allergenic residues from this source are present in the food enzyme.

The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded, but the likelihood is low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme[42]

The food enzyme is intended to be used in two food processes at the recommended use levels summarised in Table 2.

Table 2: Intended uses and recommended use levels of the food enzyme as provided by the applicant[42]

Food manufacturing process[(a)]	Raw material (RM)	Recommended dosage of the food enzyme (average – maximum) (mg TOS/kg RM)[(b)]
Modification of fats and oils by interesterification	Edible vegetable oils or edible vegetable oil fractions, free fatty acids made from edible vegetable oil	**12.5**
Manufacture of enzyme modified dairy ingredients (EMDI)	Cheese, butter and other dairy materials	187–**311**

EMDI: enzyme modified dairy ingredients; RM: Raw material; TOS: total organic solids.

(a): The description provided by the applicant has been harmonised by EFSA according to the 'EC working document describing the food processes in which food enzymes are intended to be used' – not yet published at the time of adoption of this opinion.

(b): The numbers in bold were used for calculation.

In the fat and oil modification process, vegetable oils are passed through a column packed with the immobilised food enzyme.[43] Under microaqueous environment, this triacylglycerol lipase catalyses the exchange of fatty acids at the 1- and 3-position of the triglycerides, modifying the properties of the resulting triglycerides (e.g. 2-palmitic acid enriched vegetable oils).[44] The modified fats are further incorporated into many foods as ingredients, e.g. infant formulae, croissants, doughnuts, biscuits, crackers.[45] Despite the request from EFSA, the applicant did not provide analytical data to establish the extent of possible leaching of the immobilisation agents and food enzyme–TOS into the modified fats.[15] In the absence of analytical data, the Panel decided to proceed with the dietary exposure assessment by considering that full amounts of the food enzyme–TOS remain in the modified fats.

[40] Technical dossier/p. 13, 47–48; Technical dossier/Annex 6; Technical dossier/Additional information, 14 April 2015/Answer to Point 1.

[41] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004.

[42] Technical dossier/Additional information, 4 June 2021/Answer to Point 4.

[43] Technical dossier/Additional information, 14 April 2015/p. 6–7.

[44] Technical dossier/Additional information, 4 June 2021/Answer to Point 4.1.

[45] Technical dossier/Additional information, 14 April 2015/Answer to Point 4.

To produce enzyme modified dairy ingredients (EMDI), the food enzyme is added to a variety of dairy ingredients.[42] Free fatty acids released from the hydrolysis reaction are the principle flavouring agents, resulting in EMDI products with modified sensory properties. The food enzyme remains in the EMDI products.

Based on data provided on thermostability (see Section 3.3.1), it is expected that the triacylglycerol lipase is inactivated by heat during both food manufacturing processes.

3.5.2. Dietary exposure estimation

Chronic exposure to the food enzyme–TOS was calculated by combining the maximum recommended use level with individual consumption data (EFSA CEP Panel, 2021a). The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEP Panel, 2021b). Exposure from all FoodEx categories was subsequently summed up, averaged over the total survey period (days) and normalised for bw. This was done for all individuals across all surveys, resulting in distributions of individual average exposure. Based on these distributions, the mean and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only 1 day per subject were excluded and high-level exposure/intake was calculated for only those population groups in which the sample size was sufficiently large to allow calculation of the 95th percentile (EFSA, 2011).

Table 3 provides an overview of the derived exposure estimates across all surveys. Detailed mean and 95th percentile exposure to the food enzyme–TOS per age class, country and survey, as well as contribution from each FoodEx category to the total dietary exposure are reported in Appendix A – Tables 1 and 2. For the present assessment, food consumption data were available from 43 dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure was estimated to be about 0.057 mg TOS/kg bw per day in infants at the 95th percentile.

Table 3: Summary of the estimated dietary exposure to food enzyme–TOS in six population groups

Population group	Estimated exposure (mg TOS/kg body weight per day)					
	Infants	**Toddlers**	**Children**	**Adolescents**	**Adults**	**The elderly**
Age range	3–11 months	12–35 months	3–9 years	10–17 years	18–64 years	≥ 65 years
Min–max mean (number of surveys)	0.010–0.028 (12)	0.005–0.021 (15)	0.003–0.012 (19)	0.002–0.006 (21)	0.001–0.005 (22)	0.001–0.007 (23)
Min–max 95th (number of surveys)	0.032–0.057 (11)	0.018–0.033 (14)	0.009–0.026 (19)	0.005–0.015 (20)	0.003–0.012 (22)	0.003–0.016 (22)

TOS: total organic solids.

3.5.3. Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2006), the following sources of uncertainties have been considered and are summarised in Table 4.

Table 4: Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
Model input data	
Consumption data: different methodologies/representativeness/underreporting/ misreporting/no portion size standard	+/–
Use of data from food consumption surveys of a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Possible national differences in categorisation and classification of food	+/–

Sources of uncertainties	Direction of impact
Model assumptions and factors	
In the absence of analytical data, it was assumed that the food enzyme-TOS remains fully in the modified fats obtained from immobilised enzyme.	+
Exposure to food enzyme–TOS was always calculated based on the recommended maximum use level	+
Selection of broad FoodEx categories for the exposure assessment	+
Use of recipe fractions in disaggregation FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

TOS: total organic solids.
+: uncertainty with potential to cause overestimation of exposure.
–: uncertainty with potential to cause underestimation of exposure.

The conservative approach applied to estimate the dietary exposure to the food enzyme-TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to overestimation of the exposure.

3.6. Margin of exposure

A comparison of the NOAEL (1,960 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.001–0.028 mg TOS/kg bw per day at the mean and from 0.003 to 0.057 mg TOS/kg bw per day at the 95th percentile resulted in a margin of exposure (MoE) of at least 34,386.

4. Conclusions

Based on the data provided and the derived margin of exposure for the food manufacturing processes, the Panel concluded that the food enzyme triacylglycerol lipase produced with the non-genetically modified *R. arrhizus* strain AE-TL(B) does not give rise to safety concerns under the intended conditions of use.

Documentation as provided to EFSA

Technical dossier `Application for authorisation of triacylglycerol lipase from *Rhizopus oryzae* AE-TL‘. 30 October 2013. Submitted by Amano Enzyme Inc.

Additional information. 10 July 2014. Submitted by Amano Enzyme Inc.

Additional information. 14 April 2015. Submitted by Amano Enzyme Inc.

Additional information. 4 June 2021. Submitted by Amano Enzyme Inc.

Additional information. 8 July 2022. Submitted by Amano Enzyme Inc.

Additional information. 22 December 2022. Submitted by Amano Enzyme Inc.

Summary report on technical data and dietary exposure report. 28 January 2015. Delivered by Hylobates Consulting and BiCT (Roma, Italy).

Summary report on genotoxicity, subchronic toxicity study and allergenicity report. 28 January 2015. Delivered by FoBiG (Freiburg, Germany).

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Abbreviations

A/G	albumin globulin ratio
ALT	alanine transaminase
AST	aspartate aminotransferase
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CHL/IU	Chinese hamster lung cells
EFSA CEF Panel	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
EFSA CEP Panel	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EFSA GMO Panel	EFSA Panel on genetically modified organisms
EINECS	European Inventory of Existing Commercial Chemical Substances
EMDI	enzyme modified dairy ingredients
FAO	Food and Agricultural Organization of the United Nations
FIP	Fungi-Lipase International
FoodEx	a standardised food classification and description system
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMHW	Japanese Ministry of Health and Welfare
LoQ	limit of quantification
MoE	margin of exposure
NA	not analysed
[redacted]	[redacted]
NOAEL	no observed adverse effect level
non-GM	non-genetically modified
OECD	Organisation for Economic Cooperation and Development
RM	raw material
[redacted]	[redacted]
SPF	specific pathogen free
TEU	trans-esterification units
TOS	total organic solids
WHO	World Health Organization

Appendix A – Dietary exposure estimates to the food enzyme–TOS in details

Appendix A can be found in the online version of this output (in the 'Supporting information' section). The file contains two sheets, corresponding to two tables.

Table 1: Average and 95th percentile exposure to the food enzyme–TOS per age class, country and survey.

Table 2: Contribution of food categories to the dietary exposure to the food enzyme–TOS per age class, country and survey.

Appendix B – Population groups considered for the exposure assessment

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Republic of North Macedonia*, Serbia*, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Republic of North Macedonia, Serbia*, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Bosnia and Herzegovina*, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Bosnia and Herzegovina*, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden
The elderly[(a)]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Montenegro*, Netherlands, Portugal, Romania, Serbia*, Slovenia, Spain, Sweden

*: Consumption data from these pre-accession countries are not reported in Table 3 of this opinion, however, they are included in Appendix A for testing purpose.

(a): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011).