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Safety evaluation of the food enzyme inulinase from the genetically modified *Aspergillus oryzae* strain MUCL 44346

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Abstract

The food enzyme inulinase (1-β-D-fructan fructanohydrolase; EC 3.2.1.7) is produced with the genetically modified *Aspergillus oryzae* strain MUCL 44346 by PURATOS NV. The genetic modifications do not give rise to safety concerns. The food enzyme is free from viable cells of the production organism and its DNA. It is intended to be used in the production of fructo-oligosaccharides (FOS) from inulin extracted from chicory roots. Dietary exposure to the food enzyme–total organic solids (TOS) was estimated to be up to 0.01 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 (NOAEL) of 100 mg TOS/kg bw per day, which when compared with the estimated dietary exposure, resulted in a margin of exposure of at least 10,000. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and two matches were found with tomato allergens. The Panel considered that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme, particularly in individuals sensitised to tomato, cannot be excluded. However, the likelihood of allergic reactions is expected not to exceed the likelihood of allergic reactions to tomato. As the prevalence of allergic reactions to tomato is low, also the likelihood of such reactions to occur to the food enzyme 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, inulinase, 1-β-D-fructan fructanohydrolase, EC 3.2.1.7, *Aspergillus oryzae*, genetically modified microorganism

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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.

Four applications have been introduced by the companies 'Puratos NV sa', 'Novozymes A/S', 'Meito Sangyo Co. Ltd.' and the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) for the authorisation of the food enzymes inulinase from a genetically modified strain of *Aspergillus oryzae* (strain MUCL 44346), trypsin from porcine pancreatic glands, triacylglycerol lipase from *Candida cylindracea*, and cellulase, glucanase and hemicellulase covering xylanase and mannanase from *Aspergillus niger*, respectively.

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

[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.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes inulinase from a genetically modified strain of *Aspergillus oryzae* (strain MUCL 44346), trypsin from porcine pancreatic glands, triacylglycerol lipase from *Candida cylindracea*, and cellulase, glucanase and hemicellulase covering xylanase and mannanase from *Aspergillus niger* 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 the food enzyme inulinase from the genetically modified *A. oryzae* strain MUCL 44346.

2. Data and Methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme endo-inulinase from a genetically modified *Aspergillus oryzae* strain MUCL 44346.

Additional information was requested from the applicant during the assessment process on 28 March 2019, 23 May 2019 (Addendum) and 14 April 2020 and received on 26 February 2020 and 1 March 2023 (see 'Documentation provided to EFSA').

Following the request for additional data sent by EFSA on 14 April 2020, the applicant requested a clarification teleconference on 17 September 2021, after which the applicant provided additional data on 1 March 2023.

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[4]

IUBMB nomenclature	Inulinase
Systematic name	1-β-D-fructan fructanohydrolase
Synonyms	inulase; endoinulinase
IUBMB No	EC 3.2.1.7
CAS No	9025-67-6
EINECS No	232-802-3

Inulinases hydrolyse (2 → 1)-β-D-fructosidic linkages in the storage polysaccharide inulin. The enzyme under this assessment is intended only to be used in the production of fructo-oligosaccharides (FOS) from inulin extracted from chicory roots.

3.1. Source of the food enzyme[5]

The enzyme is produced with the genetically modified filamentous fungus *A. oryzae* strain MUCL 44346, which is deposited in [redacted] with the deposit number [redacted].[6]

[4] Technical dossier/2nd submission/p. 23.
[5] Technical dossier/2nd submission/p. 30–34; Technical dossier/Additional information, 26 February 2020.
[6] Technical dossier/2nd submission/Annex 25.

The strain has been unambiguously identified as [redacted][7]. The identity of the parental strain was further demonstrated [redacted][8].

3.1.1. Characteristics of the parental and recipient microorganisms

The parental strain [redacted][9].

3.1.2. Characteristics of introduced sequences

[redacted][10], [11] from [redacted][12] [redacted][13].

3.1.3. Description of the genetic modification process

[redacted][14].

3.1.4. Safety aspects of the genetic modification

The technical dossier contains all necessary information on the recipient microorganism, the donor organism and the genetic modification process.

The production strain *A. oryzae* MUCL 44346 [redacted][15]. This is further considered in Section 3.3.4. No other issues of concern arising from the genetic modifications were identified by the Panel.

[7] Technical dossier/2nd submission/Annex 23.
[8] Technical dossier/Additional information, 26 February 2020/24022020 Round 2–2 questions.
[9] Technical dossier/2nd submission/Annex 22.
[10] Technical dossier/2nd submission/p. 66.
[11] Technical dossier/2nd submission/p. 68.
[12] Technical dossier/2nd submission/p. 69.
[13] Technical dossier/2nd submission/p. 72–73.
[14] Technical dossier/2nd submission/p. 78–81.
[15] Technical dossier/2nd submission/Annex 26.

3.2. Production of the food enzyme[16]

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[17], with food safety procedures based on Hazard Analysis and Critical Control Points,[18] and in accordance with current Good Manufacturing Practice.

The production strain is grown as a pure culture using a typical industrial medium [redacted]. The applicant provided information on the identity of the substances[19] used to control the fermentation and in the subsequent downstream processing of the food enzyme.

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[20]

The inulinase is a single polypeptide chain of [redacted] amino acids including a signal sequence of [redacted] amino acids.[21] The molecular mass of the mature protein, calculated from the amino acid sequence, is around [redacted] kDa.[22] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[23] A consistent protein pattern was observed across all batches. The gels showed a single major protein band corresponding to an apparent molecular mass of about [redacted] kDa,[24] consistent with the expected mass of the enzyme. No other enzyme activities were reported.[25]

The in-house determination of enzyme activity[26] is based on hydrolysis of chicory inulin (reaction conditions: pH 5, 50°C, 30 min). The enzymatic activity is determined by measuring the release of fructose using colorimetric assay. The enzyme activity is expressed in Units of Inulinase (UI)/mL. One UI is defined as the amount of enzyme releasing 1 μmoL of fructose equivalents per minute under the assay conditions.[26]

The food enzyme has a temperature optimum between 60 and 70°C (pH 5) and a pH optimum between 4 and 6 (65°C). Pre-incubation of the food enzyme at temperatures of 40–90°C for up to 5 h showed that the inulinase is inactivated at 70°C and above.[27]

3.3.2. Chemical parameters[28]

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation and two batches produced for the toxicological tests (Table 1). The mean total organic solids (TOS) of the three batches for commercialisation was 0.85% and the mean enzyme activity/TOS ratio was 488 UI/mg TOS.

[16] Technical dossier/2nd submission/p. 36–42; Technical dossier/2nd submission/Annex 09.
[17] 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.
[18] Technical dossier/2nd submission/p. 36; Technical dossier/2nd submission/Annex 08.
[19] Technical dossier/2nd submission/p. 37.
[20] Technical dossier/2nd submission/p. 24–30.
[21] Technical dossier/2nd submission/p. 25; Technical dossier/2nd submission/Annex 18.
[22] Technical dossier/2nd submission/p. 25–26; Technical dossier/Additional information, 1 March 2023/Annex_1_2_06.
[23] Technical dossier/2nd submission/p. 25–26; Technical dossier/2nd submission/Annex 06a; Annex 06b; Technical dossier/Additional information, 1 March 2023/Annex_1_2_06.
[24] Technical dossier/2nd submission/Annex 06a; Annex 06b; Technical dossier/Additional information, 1 March 2023/Annex_1_2_06.
[25] Technical dossier/2nd submission/p. 25; Technical dossier/Additional information, 1 March 2023.
[26] Technical dossier/2nd submission/Annex 03.
[27] Technical dossier/2nd submission/p. 30; Technical dossier/Additional information, 26 February 2020.
[28] Technical dossier/2nd submission/p. 24, 50; Technical dossier/2nd submission/Annex 01; Annex 02; Annex 03; Annex 15a; Annex 15b; Annex 15c; Annex 16; Annex 17; Technical dossier/Additional information, 26 February 2020/Annex Q1; Technical dossier/Additional information, 1 March 2023/Annex 1_1_01; Annex_1_2_01; Annex_1_2_05; Annex_1_2_04; Annex_1_2_03; Annex_1_2_02; Annex 3_01.

The batch 5 used for the *in vitro* mammalian cell micronucleus test and repeated dose 90-day oral toxicity study in rats was obtained from batch 1 by freeze drying.

Table 1: Composition of the food enzyme[29]

Parameters	Unit	Batches				
		1	2	3	4[(a)]	5[(b)]
Inulinase activity	UI/mL batch[(c)]	3,673	5,052	3,319	3,148	226,699
Protein	%	0.43	0.76	0.74	0.32	30.91
Ash	%	0.54	1.10	0.88	0.20	40.3
Water	%	98.62	98.17	98.13	98.56	0.40
Total organic solids (TOS)[(d)]	%	0.84	0.73	0.99	1.24	59.30
Activity/TOS	UI/mg TOS	437	692	335	254	382

(a): Batch used for the Ames test.
(b): Batch used for the *in vitro* mammalian cell micronucleus test and repeated dose 90-day oral toxicity study in rats.
(c): UI: Unit of Inulinase (see Section 3.3.1).
(d): TOS calculated as 100% − % water − % ash.

3.3.3. Purity[30]

The lead content[31] in the three commercial batches and in the batches used for toxicological studies was below 5 mg/kg, which complies with the specification for lead as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006).

The food enzyme 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). No antimicrobial activity was detected in any of the tested batches.[32]

Strains of *Aspergillus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites (Frisvad et al., 2018). The presence of aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, total aflatoxin, ochratoxin A, deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, total T-2/HT-2, fumonisin B1, fumonisin B2 and total fumonisins was examined in three food enzyme batches and all were below the limit of detection (LoD) of the applied methods.[33] The absence of kojic acid was confirmed in the five batches by liquid chromatography with tandem mass spectrometry (LC–MS/MS).[34] Adverse effects caused by the possible presence of other secondary metabolites was 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 and DNA of the production strain[35]

The absence of viable cells of the production strain in the food enzyme was demonstrated in three batches of the food enzyme analysed in triplicate. [redacted].[36] No colonies were produced. A positive control was included.

[29] Technical dossier/Additional information, 26 February 2020/Annex Q1; Technical dossier/Additional information, 1 March 2023/Annex 1_1_01; Annex_1_2_01; Annex_1_2_05; Annex_1_2_04; Annex_1_2_03; Annex_1_2_02; Annex 3_01.
[30] Technical dossier/2nd submission/p. 25; Technical dossier/2nd submission/Annex 04; Annex 04b; Annex 05; Technical dossier/Additional information, 1 March 2023/Annex_1_2_05; Annex_1_2_04; Annex_1_2_03; Annex_1_2_02.
[31] Technical dossier/Additional information, 1 March 2023/Annex_1_2_03; Technical dossier/2nd submission/Annex 04a/LoD for Pb = 0.10 mg/kg.
[32] Technical dossier/2nd submission/p. 9; Technical dossier/Additional information, 1 March 2023/Annex_1_2_03; Annex_1_2_04.
[33] Technical dossier/Additional information, 1 March 2023/Annex_1_2_03. LoDs for aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2 = 0.1 μg/kg, total aflatoxin = 0.4 μg/kg, ochratoxin A = < 0.2 μg/kg, deoxynivalenol = 20 μg/kg, zearalenone = 10 μg/kg, T-2 toxin = 7.5 μg/kg, HT-2 toxin = 7.5 μg/kg, total T-2/HT-2 = 15 μg/kg, fumonisin B1 = 20 μg/kg, fumonisin B2 = 20 μg/kg and total fumonisins = 40 μg/kg.
[34] Technical dossier/Additional information, 26 February 2020/Annex Q2; Limit of quantification (LoQ) for kojic acid = 5 ppm.
[35] Technical dossier/Additional information, 26 February 2020/Annex Q2; Technical dossier/Additional information, 1 March 2023/Annex 2_01; Technical dossier/2nd submission/Annex 31.
[36] Technical dossier/Additional information, 26 February 2020/Annex Q2.

The absence of recombinant DNA in the food enzyme was demonstrated [redacted] of three batches of the enzyme concentrate before formulation analysed in triplicate. [redacted], no DNA was detected [redacted],[37] with a limit of detection of 10 ng genomic DNA/mL sample.[36,38]

3.4. Toxicological data

A battery of toxicological tests, including a bacterial reverse mutation test (Ames test), an *in vitro* mammalian cell micronucleus test and a repeated dose 90-day oral toxicity study in rats, were provided.

The batches 4 and 5 (Table 1) used in these studies have similar protein pattern and a similar or lower activity/TOS values as the 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 test (Ames test) was performed according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP).[39]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA(pKM101) were used with or without metabolic activation (S9-mix), applying the 'treat and wash' method.

Two separate experiments were carried out in triplicate, using 5–7 different concentrations of the food enzyme ranging from: 5 to 5,000 μg TOS/plate in the first experiment in the presence or absence of S9-mix (10%); from 15 to 5,000 μg TOS/plate in the absence of S9-mix and from 50 to 5,000 μg TOS/plate in the presence of S9-mix (20%) in the second experiment.

In the first experiment, toxic effects, evident as thinning of the background lawn and as a reduction in the number of revertant colonies, occurred in *S*. Typhimurium strains TA100 and TA1537 at 1,500 μg TOS/plate and above, and in *S*. Typhimurium strains TA98 and TA1535 at 5,000 μg TOS/plate without S9-mix. No cytotoxicity was observed at any concentration of the test substance in *S*. Typhimurium strains with S9-mix and in *E. coli* WP2uvrA(pKM101), with or without S9-mix.

In the second experiment, toxic effects occurred in *S*. Typhimurium strains TA98, TA100 and TA1537 at 5,000 μg TOS/plate without S9-mix. No cytotoxicity was observed at any concentration of the test substance in any strain with S9-mix.

Upon treatment with the food enzyme there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix.

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

3.4.1.2. *In vitro* mammalian cell micronucleus test

The *in vitro* mammalian cell micronucleus test was carried out according to OECD Test Guideline 487 (OECD, 2016) and following GLP.[40]

An experiment was performed with duplicate cultures of mouse lymphoma L5178Y TK+/−. Cell cultures were exposed to the food enzyme and scored for the number of micronucleated cells at concentrations of 50, 100, 300 and 900 μg TOS/mL in a short-term treatment (3 h exposure and 21 h recovery period) either with or without S9-mix and at concentrations of 11.1, 25 and 50 μg TOS/mL in a long-term treatment (24 h exposure without recovery period) without S9-mix.

Marked cytotoxicity was observed at 900 μg TOS/mL (relative increase in cell counts (RICC) = 12%) in the short-term treatment with S9-mix, at 900 μg TOS/mL (RICC = 23%) in the short-term treatment without S9-mix and at 50 μg TOS/mL (RICC = 47%) in the long-term treatment without S9-mix. The number of micronucleated cells was not statistically significantly different to the negative controls at any concentrations tested.

[37] Technical dossier/2nd submission/Annex 31.
[38] Technical dossier/2nd submission/Annex 29.
[39] Technical dossier/Additional information, 26 February 2020/Annex Q3.
[40] Technical dossier/Additional information, 1 March 2023/Annex_3_02.

The Panel concluded that the food enzyme inulinase did not induce an increase in the number of micronucleated cells 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 followed OECD Test Guideline 408 (OECD, 2018) and GLP.[41]

Groups of 10 male and 10 female Crl:WI (Wistar) rats received by gavage the food enzyme in doses of 100, 300 or 1,000 mg TOS/kg bw per day. Controls received the vehicle (distilled water).

No mortality was observed.

Haematological investigations revealed a statistically significant increase in red blood cell count in high-dose females (+10%), an increase in haematocrit in high-dose females (+6%), an increase in red cell distribution width (RDW) in mid- and high-dose females (+5% and +6%, respectively), a decrease in mean corpuscular haemoglobin in high-dose females (−5%) and a decrease in relative basophils (Ba%) in high-dose females (−64%). The Panel considered the changes as not toxicologically relevant, as they were only observed in one sex (all parameters), the changes were small (all parameters), there were no changes in other relevant parameters (for Ba% in total and differential count of white blood cells), there were no histopathological changes in bone marrow and the changes were within the historical control values (except for RDW).

Clinical chemistry investigations revealed a statistically significant increase in total bilirubin (TBIL) and bile acids (BA) concentrations in high-dose males (+36% and +75%, respectively), an increase in albumin/globulin ratio (A/G) in low- and mid-dose males (+6% and +7%, respectively), a dose-dependent increase in TBIL in low-, mid- and high-dose females (+16%, +32% and +87%, respectively, reaching statistical significance in mid- and high-dose groups), an increase in BA in high-dose females (+288%), an increase in cholesterol (Chol) in high-dose females (+28%), a decrease in A/G in high-dose females (−10%), an increase in alkaline phosphatase (ALKP) activity in mid- and high-dose females (+42% and +48%, respectively), a dose-dependent increase in triglycerides (TRIG) in low-, mid- and high-dose females (+80%, +153% and +522%, respectively, reaching the statistical significance in mid- and high-dose groups) and a dose-dependent increase in low-density lipoprotein (LDL) in low-, mid- and high-dose females (+15%, +25% and +68%, respectively, reaching statistical significance in high-dose group). The Panel considered the changes in TBIL and BA, TRIG, and ALKP as indicative of altered liver function. The Panel considered the other changes as not toxicologically relevant as they were only observed in one sex (Chol, LDL), the changes were small (A/G), there was no consistency between males and females (A/G), there were no changes in other relevant parameters (for A/G in albumin, globulin) and the changes were within the historical control values (all parameters, except of BA and TRIG in high-dose females).

Statistically significant changes in organ weights included an increase in the absolute liver weight in high-dose males and females (+18% and +43%, respectively), an increase in the relative liver to body weight in high-dose males and females (+20% and +34%, respectively), an increase in the relative liver to brain weight in high-dose males and females (+16% and +40%, respectively), an increase in the absolute spleen weight in high-dose females (+30%), an increase in the relative spleen to body weight in high-dose males and females (+11% and +21%, respectively), an increase in the relative spleen to brain weight in high-dose females (+27%) and an increase in absolute heart weight in high-dose females (+10%). The Panel considered the change in heart weight as not toxicologically relevant as it was only observed in one sex, there were no histopathological changes in the heart and the change was within the historical control values. The changes in liver and spleen weights were considered toxicologically relevant as they were observed in both sexes and there were histopathological changes in these organs.

The microscopic examination revealed periportal hepatocellular vacuolation, typically with microvesicular appearance in the liver of mid- and high-dose males (1/10 [severity mild] and 4/10 [severity minimal in 3/10 and moderate in 1/10] vs. 0/10 in the control) and in mid- and high-dose females (2/10 [severity minimal] and 10/10 [severity minimal in 4/10, mild in 4/10, moderate in 1/10 and marked in 1/10] vs. 0/10 in the control, respectively). The incidence of the change was statistically significantly increased in high-dose males and females. The Panel considered that the periportal hepatocellular vacuolation could account for the increase in liver weight (absolute and relative) and it was associated with an increase in bilirubin, bile acids, triglycerides and alkaline phosphatase. Increases in bilirubin and bile acids were considered indicative for cholestasis, possibly due to a

[41] Technical dossier/Additional information, 1 March 2023/Annex_3_03.

mechanical obstruction of bile ducts in the portal area. Furthermore, an increased incidence of minimal extramedullary haematopoiesis in spleen was reported in high-dose females (6/10 vs. 3/10 in the control), but the difference to control was not statistically significant. The Panel noted that some degree of extramedullary haematopoiesis is normally present in the rat spleen, because this organ shares haematopoietic function with the bone marrow in the rat. Although extramedullary haematopoiesis can be increased in response to, e.g., haematotoxic insult, the minimal severity of this change in the control and the treated animals supports that this change is not test item related.

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

The Panel identified a no observed adverse effect level (NOAEL) of 100 mg TOS/kg bw per day, based on the changes in clinical chemistry, liver weight changes and histopathological findings in the liver in mid- and high-dose males and females.

3.4.3. Allergenicity[42]

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

The potential allergenicity of the enzyme inulinase produced with the genetically modified *A. oryzae* strain MUCL 44346 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 higher than 35% identity in a sliding window of 80 amino acids as the criterion, two matches were found. The matching allergens, those two sequences are linked to the tomato (*Solanum lycopersicum*) (gi|18542113|gb|AAL75449.1|AF465612_1 and gi|18542115|gb|AAL75450.1|AF465613_1) and described as minor allergens.[43]

There is no information on allergenicity of this inulinase produced with the genetically modified *A. oryzae* strain MUCL 44346.

The sequence homology of this enzyme with two sequences of tomato indicates a potential cross-reactivity of the enzyme with the allergen from tomato. Tomato is one of the most frequently consumed vegetables worldwide. Although a number of specific allergen proteins has been identified in tomato, genuine tomato allergy is rare (Asero et al., 2008, 2010).

[redacted], a known source of allergens, is present in the media fed to the microorganisms. However, during the fermentation process, this product 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 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, particularly in individuals sensitised to tomato, cannot be excluded. However, the likelihood of allergic reactions to the inulinase produced with the genetically modified *A. oryzae* strain MUCL 44346 is expected not to exceed the likelihood of allergic reactions to tomato. As the prevalence of allergic reactions to tomato is low, the likelihood of such reaction to occur to the food enzyme also is low.

3.5. Dietary exposure[44]

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in the production of FOS from chicory roots at the maximum recommended use levels of 2,750 IU/kg inulin, corresponding to 5.6 mg TOS/kg inulin.[45]

The food enzyme is added to purified and concentrated inulin extracted from chicory roots, which results in the conversion of inulin to FOS through partial hydrolysis.[46] The resulting product is

[42] Technical dossier/2nd submission/Annex 18.
[43] Technical dossier/2nd submission/p. 52–54.
[44] Technical dossier/2nd submission/p. 44–45.
[45] Technical dossier/Additional information, 1 March 2023/p. 10/Table 3.
[46] Technical dossier/2nd submission/p. 43/Figure 04 and p. 46/Table 05; Technical dossier/Additional information, 26 February 2020/Answer 1.4.

concentrated at temperatures above 95°C, and two types of FOS products can be produced, a syrup, containing 75% dry solids or a powder following spray drying.[47]

The applicant did not provide experimental data to establish the extent of TOS carry-over into the final FOS products.[47] Based on the information about the manufacturing process of FOS from the chicory roots, it is expected that the food enzyme–TOS remains in the FOS.

Based on the reported thermal treatment during the concentration step of the product, it is anticipated that the food enzyme is inactivated. This assumption is supported by measurements of the enzymatic activity in the resulting FOS syrup (i.e., 1 UI/mL in all three batches tested).[48]

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 body weight. 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 2 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 41 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B). The highest dietary exposure to the food enzyme-TOS was estimated to be 0.01 mg TOS/kg bw per day in infants at the 95th percentile.

Table 2: 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)	0.001–0.004 (11)	0–0.002 (15)	0–0 (19)	0–0 (21)	0–0 (22)	0–0 (22)
Min–max 95th percentile (number of surveys)	0.004–0.010 (9)	0.001–0.004 (13)	0–0.001 (19)	0–0 (20)	0–0 (22)	0–0 (21)

TOS: total organic solids.

3.5.3. Uncertainty analysis

In accordance with the guidance provided in the 'EFSA Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment' (EFSA, 2007), the following sources of uncertainties have been considered and are summarised in Table 3.

[47] Technical dossier/Additional information, 26 February 2020/Answer 1.4.
[48] Technical dossier/Additional information, 26 February 2020/Annex Q1.4.

Table 3: 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	+/–
Model assumptions and factors	
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 to disaggregate FoodEx categories	+/–
Use of technical factors in the exposure model	+/–

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

The conservative approach applied to the estimate of exposure to food enzyme–TOS, in particular, assumptions made regarding 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 (100 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.000–0.004 mg TOS/kg bw per day at the mean and from 0.000–0.010 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure (MoE) of at least 10,000.

4. Conclusions

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme inulinase produced with the genetically modified *A. oryzae* strain MUCL 44346 does not give rise to safety concerns under the intended conditions of use.

The CEP Panel considered the food enzyme free from viable cells of the production organism and recombinant DNA.

5. Documentation as provided to EFSA

Technical dossier 'Application for authorisation of endo-inulinase from a genetically modified strain of *Aspergillus oryzae* MUCL 44346 in accordance with Regulation (EC) No 1331/2008'. 9 February 2015. Submitted by Puratos NV, Belgium.

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Abbreviations

A/G	albumin/globulin ratio
ALKP	alkaline phosphatase
AMFEP	Association of Manufacturers and Formulators of Enzyme Products
[redacted]	[redacted]
BA	bile acids
Ba%	relative basophils
bp	base pair
bw	body weight
CAS	Chemical Abstracts Service
[redacted]	[redacted]
Chol	cholesterol
DNA	deoxyribonucleic acid
EC	European Commission
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
EU	European Union
FAO	Food and Agriculture Organization of the United States
FoodEx	a standardised food classification and description system
FOS	fructo-oligosaccharides
GLP	Good Laboratory Practice
GM	genetically modified
GMO	genetically modified organism
IU	Unit
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
L5178Y TK+/−	mouse lymphoma cells
LC–MS/MS	liquid chromatography with tandem mass spectrometry
LDL	low-density lipoprotein
LoD	limit of detection
LoQ	limit of quantification
MoE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
[redacted]	[redacted]
RDW	red cell distribution width
RICC	relative increase in cell counts
[redacted]	[redacted]
TBIL	total bilirubin
TOS	Total Organic Solids
TRIG	triglycerides
UI	Unit of Inulinase
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
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, Slovenia, Spain
Children[(a)]	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, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, 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, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

(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).