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Safety evaluation of the food enzyme glucan-1,4-α-glucosidase from the non-genetically modified *Rhizopus delemar* strain CU634-1775

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Abstract

The food enzyme glucan-1,4-α-glucosidase (4-α-D-glucan glucohydrolase; EC 3.1.2.3) is produced with the non-genetically modified *Rhizopus delemar* strain CU634-1775 by Shin Nihon Chemical Co., Ltd. The food enzyme is free from viable cells of the production strain. It is intended to be used in six food manufacturing processes: baking processes, starch processing for production of glucose syrups and other starch hydrolysates, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, brewing processes and distilled alcohol production. As residual amounts of total organic solids (TOS) are removed by distillation and by the purification steps applied during the production of glucose syrups, dietary exposure was not calculated for these two food processes. For the remaining four food processes, dietary exposure to the food enzyme-total organic solids was estimated to be up to 1.238 mg TOS/kg body weight (bw) per day. Genotoxicity tests did not raise a safety concern. 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 1,735 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 1,401. In a search for the similarity of the amino acid sequence of the food enzyme to known allergens, a single match with a respiratory allergen was found. The Panel considered that, under the intended conditions of use, the risk of allergic reactions by 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.

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Keywords: food enzyme, glucan-1,4-α-glucosidase, 4-α-D-glucan glucohydrolase, glucoamylase, EC 3.2.1.3, *Rhizopus oryzae*

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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 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:

i) it does not pose a safety concern to the health of the consumer at the level of use proposed;
ii) there is a reasonable technological need;
iii) 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, 2009a) 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 on food enzymes.

Five applications have been introduced by the companies "Intertek Scientific & Regulatory Consultancy" for the authorisation of the food enzymes Catalase from *Aspergillus niger* (strain CTS 2093), Glucose oxidase from *Penicillium chrysogenum* (strain PGO 19–162), Tannase from *Aspergillus oryzae* (strain TAN 206) and Glucoamylase from *Rhyzopus oryzae* (strain CU634-1775), and "RDA Scientific Consultants GmbH" for the authorisation of the food enzyme Phospholipase D from *Streptomyces netropsis* (DSZM No. 40093).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011[3] implementing Regulation (EC) No 1331/2008, the Commission has verified that the five 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, 11.03.2011, 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 Catalase from *Aspergillus niger* (strain CTS 2093), Glucose oxidase from *Penicillium chrysogenum* (strain PGO 19–162), Tannase from *Aspergillus oryzae* (strain TAN 206), Glucoamylase from *Rhyzopus oryzae* (strain CU634-1775) and Phospholipase D from *Streptomyces netropsis* (DSZM No. 40093) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 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 glucoamylase from *Rhyzopus oryzae* strain CU634-1775. Recent data identified the production microorganism as *Rhizopus delemar* (Section 3.1). Therefore, this name will be used in this opinion instead of *Rhyzopus oryzae*.

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme glucoamylase from *Rhizopus oryzae* strain CU634-1775.

Additional information was requested from the applicant during the assessment process on 15 January 2020, 15 February 2021 and 17 March 2021 and was consequently provided (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, 2009b) 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, 2009a) 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

IUBMB nomenclature: Glucan 1,4-α-glucosidase
Systematic name: 4-α-D-glucan glucohydrolase
Synonyms: Glucoamylase
IUBMB No.: EC 3.2.1.3
CAS No.: 9032-08-0
EINECS No.: 232-877-2

Glucan-1,4-α-glucosidases catalyse the hydrolysis of terminal (1–4)-linked α-D-glucose residues successively from non-reducing ends of amylopectin and amylose with the release of glucose. The enzyme under assessment is intended to be used in six food manufacturing processes: baking processes, starch processing for production of glucose syrups and other starch hydrolysates, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, brewing processes and distilled alcohol production.

3.1. Source of the food enzyme

The glucan 1,4-α-glucosidase is produced with the non-genetically modified filamentous fungus *Rhizopus delemar* strain CU634-1775, which is deposited at the CAB International Genetic Resource Collection (UK) with the deposit number SD152. The production strain was identified as *R. delemar* (formerly considered as a synonym of *R. oryzae*) by [redacted].[4] *R. delemar* CU634-1775 was isolated from food.

[4] Technical dossier/Additional data 30 March 2021/Attachment A.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No. 852/2004[5], with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.

The production strain is grown as a pure culture using a typical industrial medium in a [redacted] fermentation system with conventional process controls in place. After completion of the fermentation, the food enzyme is extracted from the fermentation medium and then the solid biomass is removed from the extract by centrifugation and microfiltration. The filtrate containing the enzyme is further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained, while most of the low molecular mass material passes the filtration membrane and is discarded.[6] 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.[7]

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 glucan 1,4-α-glucosidase is a single polypeptide chain of [redacted] amino acids.[8] The molecular mass of the mature protein, calculated from the amino acid sequence, is 65 kDa. The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. 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 71.8 kDa, consistent with the expected mass of the glycosylated enzyme. The protein profile also included bands of lower staining intensity.[9] No other enzyme activities were reported.

The in-house determination of glucan 1,4-α-glucosidase activity is based on hydrolysis of starch, (reaction conditions: pH 4.5, 40°C, 10 min). The enzymatic activity is determined by measuring the release of glucose by titration using a modified Fehling-Lehman-Schoorl method. The enzyme activity is measured in Units (U)/g. One U is defined as the quantity of enzyme that liberates 1 mg of reducing sugar expressed as D-glucose per minute under the condition of the assay.[10]

The food enzyme has a temperature optimum around 60°C (pH 4.5) and a pH optimum around pH 5 (40°C). Thermostability was tested after a pre-incubation of the food enzyme for 15 min at different temperatures (pH 4.5). Glucan 1,4-α-glucosidase activity decreased above 40°C, showing no residual activity above 70°C.[11]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches used for commercialisation (1–3) and two batches (4 and 5) produced for the toxicological tests (Table 1).[12] The mean total organic solids (TOS) of the three food enzyme batches for commercialisation was 17.2% and the mean enzyme activity/TOS ratio was 10.4 U/mg TOS.

[5] 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.
[6] Technical dossier/pp. 35–37.
[7] Technical dossier/p. 35.
[8] Technical dossier/p. 19.
[9] Technical dossier/pp.18 and 20.
[10] Technical dossier/Annex II.1.
[11] Technical dossier/pp. 23–27.
[12] Technical dossier/Annexes IV and V.

Table 1: Compositional data of the food enzyme

Parameter	Unit	Batches				
		1	2	3	4[(a)]	5[(b)]
Glucan 1,4-α-glucosidase activity	U/g batch[(c)]	1,750	1,720	1,860	1,790	1,700
Protein	%	11.0	10.3	12.5	12.8	10.5
Ash	%	0.6	0.3	0.4	0.4	0.7
Water	%	81.0	83.7	82.5	83.5	81.9
Total organic solids (TOS)[(d)]	%	18.4	16.0	17.1	16.1	17.4
Activity/TOS	U/mg TOS	9.5	10.8	10.9	11.1	9.8

(a): Batch used for the second bacterial reverse mutation test and the *in vivo* gene mutation assay in transgenic mice.
(b): Batch used for the first bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, *in vitro* mammalian cell gene mutation test with mouse lymphoma cells and 90-day oral toxicity studies in rodents.
(c): U: Glucan 1,4-α-glucosidase units (see Section 3.3.1).
(d): TOS calculated as 100% − % water − % ash.

3.3.3. Purity

The lead content of the three commercial batches and the two batches used for toxicological studies was below 0.05 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). In addition, the levels of arsenic were below 0.1 mg/kg.[13,14]

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.[15]

Strains of *Rhizopus*, in common with most filamentous fungi, have the capacity to produce a range of secondary metabolites. The presence of aflatoxins (B1, B2, G1 and G2), ochratoxin A, sterigmatocystin, T-2 toxin and zearalenone was examined in three food enzyme batches and all were below the limits of detection (LoD) of the applied analytical methods.[16] Adverse effects caused by the possible presence of other secondary metabolites of concern are addressed by the toxicological examination of the food enzyme–TOS.

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

3.3.4. Viable cells of the production strain

The absence of the production strain in the food enzyme was demonstrated [redacted]. No colonies were produced. A positive control was included.[17]

3.4. Toxicological data

A battery of toxicological tests, including two bacterial gene mutation assays (Ames tests), an *in vitro* mammalian chromosomal aberration test, an *in vitro* gene mutation assay in mouse lymphoma cells, an *in vivo* gene mutation assay in transgenic mice and a repeated dose 90-day oral toxicity studies in rats, has been provided. The batches 4 and 5 (Table 1) used in these studies have similar protein pattern as the batches used for commercialisation and, thus, are considered suitable as test items.

[13] Technical dossier/Additional data January 2020/Attachment A.
[14] LoDs: Pb = 0.05 mg/kg; As = 0.1 mg/kg.
[15] Technical dossier/Annex IV.1.
[16] LoDs: aflatoxins B1, B2, G1 and G2 = 0.5 μg/kg each; ochratoxin A = 0.5 μg/kg, sterigmatocystin = 20 μg/kg; T-2 toxin = 0.1 mg/kg; zearalenone = 50 μg/kg.
[17] Technical dossier/ Additional data January 2021 and Additional data 2 March 2021/Attachment A.

3.4.1. Genotoxicity

3.4.1.1. *In vitro* studies

3.4.1.1.1. Bacterial reverse mutation tests

First study (batch 5)

The first bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).[18]

Four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* WP2uvrA were used in the presence or absence of metabolic activation (S9-mix). A concentration-range finding test applying the standard preincubation method was carried out in triplicate using eight concentrations of food enzyme (0.0079, 0.024, 0.071, 0.21, 0.64, 1.9, 5.8 and 17 mg TOS/plate). In the absence of S9-mix, a significant increase in revertant colony numbers above the control values were observed in *S*. Typhimurium TA98 (at 17 mg TOS/plate) in TA1535 and TA1537 (at 5.8 and 17 mg TOS/plate). In the presence of S9-mix, a significant increase in revertant colony numbers above the control values were observed in TA100 (at 17 mg TOS/plate), and in TA98 and TA1535 (at 5.8 and 17 mg TOS/plate).

Subsequently, a test applying a pre-incubation method was conducted in triplicate in *S*. Typhimurium TA100 in the absence of S9-mix, in TA1537 in the presence of S9-mix and in *E. coli* WP2uvrA in the presence and absence of S9-mix. Seven concentrations of the food enzyme (0.27, 0.54, 1.1, 2.2, 4.3, 8.7 and 17 mg TOS/plate) were tested. No significant increase in revertant colony numbers above the control values was observed in any strain with or without S9-mix. However, in both tests, an increase in the growth of the background lawn was observed, which was indicative of the presence of free amino acids, such as histidine, in the test substance. Therefore, another main test and a confirmatory test applying a modified treat and plate assay were conducted.

The main test applying the modified treat and plate assay was carried out in triplicate, using eight different concentrations of food enzyme (0.0079, 0.024, 0.071, 0.21, 0.64, 1.9, 5.8 and 17 mg TOS/plate). Three strains of *S*. Typhimurium (TA1535, TA98 and TA1537) in the absence of S9-mix and three strains of *S*. Typhimurium (TA100, TA1535 and TA98) in the presence of S9-mix were used. Upon treatment with the food enzyme, a significant increase in revertant colony numbers above the control values (2.6–2.8 fold) was observed in the TA1537 strain without S9-mix at the highest concentrations (1.9, 5.8, and 17 mg TOS/plate). The confirmatory test applying the modified treat and plate assay was carried out in triplicate. In the absence of S9-mix, eight concentrations of food enzyme (0.14, 0.27, 0.54, 1.1, 2.2, 4.3, 8.7 and 17 mg TOS/plate) were tested in three strains of *S*. Typhimurium (TA1535, TA98 and TA1537). In the presence of S9-mix, six concentrations of food enzyme (0.54, 1.1, 2.2, 4.3, 8.7 and 17 mg TOS/plate) were tested in three strains of *S*. Typhimurium (TA100, TA1535 and TA98). Upon treatment with the food enzyme, a significant increase in revertant colony numbers above the control values (2.2–2.6 fold) was observed at two concentrations (4.3 and 17 mg TOS/plate) in the TA1537 strain without S9-mix.

A second confirmatory test applying the modified treat and plate assay (restudy) in the absence of S9-mix was carried out in triplicate using eight different concentrations of food enzyme (0.14, 0.27, 0.54, 1.1, 2.2, 4.3, 8.7 and 17 mg TOS/plate) in *S*. Typhimurium TA1537. A significant increase in revertant colony numbers above the control values (2.2 fold) was observed at the highest concentration of 17 mg TOS/plate.

The Panel concluded that the food enzyme induced gene mutations under the test conditions employed in this study.

Second study (batch 4)

The second bacterial reverse mutation assay (Ames test) was performed according to the OECD Test Guideline 471 (OECD, 1997a) and following GLP.[19] Four strains of *Salmonella* Typhimurium (TA100, TA98, TA1535 and TA1537) and *Escherichia coli* (WP2uvrA) were used in the presence or absence of metabolic activation (S9-mix), applying a pre-incubation method.

Two separate experiments were carried out in duplicate using eight concentrations of the food enzyme in the concentration-finding study (7.36, 22.1, 66.3, 199, 596, 1,790, 5,370 and 16,100 μg

[18] Technical dossier/Annex VI/1.
[19] Technical dossier/Annex VI/2.

TOS/plate) and in the main test with *E. coli*. Six concentrations of the food enzyme (66.3, 199, 596, 1,790, 5,370 and 16,100 μg TOS/plate) were applied in *S*. Typhimurium strains in the main study wAn accelerated growth of background bacteria was observed at higher doses in each strain. No cytotoxicity was observed at any concentration level of the test substance. 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 did not induce gene mutations under the test conditions employed in this study.

3.4.1.1.2. In vitro *gene mutation assay with mouse lymphoma cells*

The *in vitro* gene mutation assay was carried out in mouse lymphoma cells (L5178Y $tk^{+/-}$ 3.7.2C) (Mouse Lymphoma Assay) according to OECD Test Guideline 476 (OECD, 1997b) and following GLP.[20]

Based on the results of a dose-finding study, the cells were exposed at nine concentrations of food enzyme from 0.664 to 170 U/mL (corresponding to 0.068, 0.14, 0.27, 0.54, 1.08, 2.17, 4.34, 8.67 and 17.3 mg TOS/mL) for the short-term (3 h) treatment with and without metabolic activation (S9-mix), and at 10 concentrations of food enzyme from 6.86 to 170 U/mL (corresponding to 0.7, 1.0, 1.43, 2.04, 2.92, 4.16, 5.95, 8.5, 12.14 and 17.35 mg TOS/mL) for the continuous treatment (24 h) in the absence of S9-mix.

No precipitation of the test substance was observed at any concentration at the start and end of treatment. The relative total growth (RTG), an indicator of cytotoxicity, was 10% or less at the concentration of 170 U/mL in short-term treatment in the absence of S9-mix.

The mutant frequency was calculated on day 2 after treatment. A statistically significant and concentration-related increase in mutant frequency (MF) was observed in the short-term treatment in the presence and absence of metabolic activation and in the continuous treatment in the absence of metabolic activation. The MF exceeded the threshold of 126 (global evaluation factor according to OECD Test Guideline 490) above the corresponding solvent control at the highest concentrations tested in the short-term treatment with and without S9 mix.

The Panel concluded that food enzyme induces gene mutation in mouse lymphoma cells under the test conditions employed for this study.

3.4.1.1.3. In vitro *mammalian chromosomal aberration test*

The *in vitro* mammalian chromosomal aberration test was carried out in Chinese hamster lung fibroblast cell lines (CHL/IU) according to OECD Test Guideline 473 (OECD, 1997c) and following GLP.[21]

Based on the results of the cell growth inhibition test, the cells were exposed to the food enzyme at concentrations of 42.5, 85 and 170 U/mL, corresponding to 4.3, 8.7 and 17.3 mg TOS/mL, in a short-term treatment (6 h followed by 18 h recovery period) with and without metabolic activation (S9-mix), and in a continuous treatment (24 h) in the absence of S9-mix. Only a slight decrease in relative growth rate (79.7%) was observed after 24 h continuous treatment in the absence of S9-mix. The frequency of structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls (physiological saline) and was within the range of the laboratory historical solvent control data.

The Panel concluded that food enzyme did not induce chromosomal aberrations under the test conditions employed for this study.

3.4.1.2. *In vivo* studies

3.4.1.2.1. Gene mutation assay in transgenic mice

A gene mutation assay with transgenic mice strain C57BL/6JJmsSlc-Tg (*gpt* delta) [SPF], was carried out according to OECD Draft Guideline 488 (OECD, 2013) and following GLP.[22] The assay was conducted to assess the potential of the food enzyme to induce gene mutation (reporter gene: *gpt* and *red/gam*) in the target organs (liver and stomach) *in vivo*.

Groups of six male transgenic mice received by gavage the food enzyme in doses of 403, 805 and 1,610 mg TOS/kg body weight (bw) per day for 28 consecutive days. Three days after the final treatment, the liver and stomach were removed and mutant frequencies in these organs were determined. Controls (6 males) received the vehicle (water for injection).

[20] Technical dossier/Annex VI/4.
[21] Technical dossier/Annex VI/3.
[22] Technical dossier/Addendum/7.

No clinical signs, body weight changes, macroscopic findings in the liver or stomach, and differences in the liver or relative liver weights in treated mice in comparison with control mice were recorded.

No statistically significant increases in mutant frequencies (6-thioguanidine and Spi$^-$ selection) were observed in the liver or stomach in treated mice, as compared to the negative control group.

A positive control group (6 males) was treated with benzo[*a*]pyrene at the dose of 125 mg/kg per day for five consecutive days via oral gavage and showed statistically significant increase in mutant frequencies in both organs and in both assays (*gtp* and Spi$^-$).

The Panel concluded that under the test conditions employed in this study the food enzyme did not induce an increase in gene mutations in transgenic mice.

3.4.1.3. Conclusions on genotoxicity

The food enzyme glucan 1,4-α-glucosidase was tested in a battery of *in vitro* and *in vivo* genotoxicity assays, performed according to GLP and OECD guidelines. Positive results with Ames test were obtained in a first study, but were not reproduced in the second study. Increases in mutation frequency were also observed in mammalian cells with the Mouse Lymphoma Assay. Negative results were obtained in an *in vitro* chromosomal aberration test. As *in vivo* follow-up, a gene mutation assay in transgenic mice was carried out. The negative results of this study exclude the concern for gene mutations. The Panel concluded that the food enzyme did not induce gene mutations and chromosomal aberrations.

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

The repeated dose 90-day oral toxicity study was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP.[23] Groups of 10 male and 10 female Crl:CD(SD)IGS rats received by gavage the food enzyme in 170, 1,700 and 17,000 U/kg per day, corresponding to 17.3, 173 and 1,735 mg TOS/kg bw per day, for 91 consecutive days. Controls received the vehicle (water for injection).

No mortality was observed.

The feed consumption was statistically significantly decreased on day 71 in high-dose females (−11%) and increased on day 78 in mid-dose females (+5%). The feed efficiency was statistically significantly decreased (−27%) in high-dose males on day 78. The Panel considered these changes as not toxicologically relevant as they were only recorded sporadically (both parameters), they were only observed on one sex (both parameters), there was no dose–response relationship (feed intake in mid-dose females), the change was small (feed intake in mid-dose females) and these changes were without statistically significant changes on the final body weight and the final body weight gain.

The haematological investigation revealed a statistically significantly increased differential count of large unstained cells in high-dose females (+50%). The Panel considered this change as not toxicologically relevant as there were no changes in other relevant parameters (i.e. in total white blood cell count and in counts of other populations of leukocytes) and the change was only observed in one sex.

The clinical chemistry investigation revealed a dose-dependent decrease in the triglyceride concentration, which reached a statistical significance in high-dose males (−37%), and a statistically significantly increase in creatinine concentration in the low-dose males (+13%). The Panel considered these changes as not toxicologically relevant as they were only observed in one sex (both parameters), there was no dose–response relationship (creatinine) and the change in triglyceride was not considered adverse.

The urinalysis revealed an increased number of samples with no mucous threads in the urinary sediment in the treated groups (males: 1, 3, 4 and 10; females: 3, 6, 3 and 10 in the control, low-, mid- and high-dose groups, respectively). The Panel considered these changes as not toxicologically relevant as there was no dose–response relationship (females) and there were no changes in other urinalysis parameters.

Statistically significant changes in organ weights were limited to a decrease in the relative kidney weight in mid-dose females (−10%). The Panel considered this change as not toxicologically relevant as it was only observed in one sex, the change was small, there was no dose–response relationship and there were no histopathological changes in the organ.

[23] Technical dossier/Annex VI/6.

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

The Panel identified the no observed adverse effect level (NOAEL) of 1,735 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

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

The potential allergenicity of the glucan 1,4-α-glucosidase produced with the *R. delemar* strain CU634-1775 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, one match was found.[24] The matching allergen was Sch c 1, a glucoamylase produced by *Schizophyllum commune*.

No information is available on oral and respiratory sensitisation or elicitation reactions of this glucan 1,4-α-glucosidase.

Glucoamylase from *S. commune* (Toyotome et al., 2014) is known as an occupational respiratory allergen associated with baker's asthma. However, several studies have shown that adults with occupational asthma may be able to ingest respiratory allergens without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia et al., 2009).

[redacted], a substance that may cause allergies or intolerances (listed in Regulation (EU) No 1169/2011[25]), is used as a raw material in the media used to produce the microorganism. 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 concluded that, under the intended conditions of use, the risk of allergic reactions upon dietary exposure to this food enzyme cannot be excluded (except for distilled alcohol production), but the likelihood is low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in six food manufacturing processes. Intended uses and the recommended use levels are summarised in Table 2.

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

Food manufacturing process[(a)]	Raw material (RM)	Recommended dosage of the food enzyme (mg TOS/kg RM)[(b)]
Baking processes	Flour	**20**
Starch processing for production of glucose syrups and other starch hydrolysates	Corn starch	250
	Oat, wheat, barley	200
Fruit and vegetable processing for juice production	Fruit, vegetable	**20**

[24] Technical dossier/pp. 57–58 and Annex VII.1

[25] 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.

Food manufacturing process[(a)]	Raw material (RM)	Recommended dosage of the food enzyme (mg TOS/kg RM)[(b)]
Fruit and vegetable processing for products other than juices	Fruit, vegetable	**20**
Brewing processes	Malt	**250**
	Rice	200
Distilled alcohol production	Rice, barley	200

(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 are used for calculations.
(c): Technical dossier/Additional data January 2021/Answer 9.

In baking processes, the food enzyme is added to flour during dough or batter preparation.[26] The glucan 1,4-α-glucosidase releases glucose from starch, which may be fermented by yeast. The food enzyme–TOS remains in bakery foods.

In starch processing for the production of glucose syrups, the food enzyme is added during the saccharification step, where it releases glucose from starch polysaccharides.[27] The food enzyme–TOS is removed from the final glucose syrups by treatment with activated charcoal or similar, and with ion-exchange resins. This conclusion is extended to other starch hydrolysates (EFSA CEP Panel, 2021b). Heating the glucan 1,4-α-glucosidase treated syrups would give rise to caramel flavour in these syrups.[28]

In fruit and vegetable processing for juice production, the food enzyme is added to the crushed fruits or vegetables during mash treatment together with other cell-wall degrading enzymes.[29] It hydrolyses starch in the juice, reducing the turbidity. The food enzyme–TOS remains in the juices.

For other fruit and vegetable products, the food enzyme is also added to the crushed fruits or vegetables during mash treatment together with other cell-wall degrading enzymes.[30] It hydrolyses starch in the mash. The food enzyme–TOS remains in the final products (e.g. puree, tomato paste).

In brewing processes, the food enzyme is added to barley or other cereals during the mashing, saccharification and fermentation steps,[31] where it will hydrolyse the starchy content of the mash to release glucose for fermentation. The food enzyme TOS remains in the final foods (e.g. beer, sake).

In distilled alcohol production, the food enzyme is added to starch-rich plant materials (e.g. rice, sweet potato) during the fermentation steps.[32] It converts liquefied starch into a glucose-rich solution, increasing the amounts of fermentable sugars to produce alcohol. The food enzyme TOS is not carried over with the distilled alcohols (EFSA CEP Panel, 2021b).

Based on data provided on thermostability (see Section 3.3.1), it is expected that the glucan 1,4-α-glucosidase would be inactivated by heat in most of the food processes, but may remain active in juices, depending on the pasteurisation conditions.

3.5.2. Dietary exposure estimation

A dietary exposure was calculated only for food manufacturing processes where the food enzyme-TOS remains in the final foods: baking processes, fruit and vegetable processing for juice production, fruit and vegetable processing for products other than juices, and brewing processes.

Chronic exposure to the food enzyme-TOS was calculated by combining the maximum recommended use level provided by the applicant with the individual data from the EFSA Comprehensive European Food Consumption Database. The estimation involved selection of relevant food categories and application of technical conversion factors (EFSA CEF 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

[26] Technical dossier/Additional data January 2021/Attachment B-1.
[27] Technical dossier/Additional data January 2021/Attachment B-2.
[28] Technical dossier/Additional data January 2021/Attachment B-10.
[29] Technical dossier/Additional data January 2021/Attachment B-3.
[30] Technical dossier/Additional data January 2021/Attachment B-4.
[31] Technical dossier/Additional data January 2021/Attachments B-5, B-6, B-8, B-9.
[32] Technical dossier/Additional data January 2021/Attachment B-7.

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 average 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 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 1.238 mg TOS/kg bw per day in adults at the 95th percentile.

Table 3: Summary of 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)	0.051–0.293 (12)	0.132–0.671 (15)	0.124–0.374 (19)	0.062–0.260 (21)	0.074–0.316 (22)	0.039–0.205 (23)
Min–max 95th percentile (number of surveys)	0.134–0.737 (11)	0.382–0.971 (14)	0.252–0.992 (19)	0.135–0.764 (20)	0.229–1.238 (22)	0.127–0.662 (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	+/–
Model assumptions and factors	
Exposure to food enzyme–TOS 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	+/–
Exclusion of two processes from the exposure assessment: Starch processing for production of glucose syrups and other starch hydrolysates, distilled alcohol production	–

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 the exposure estimate to food enzyme–TOS, in particular assumptions made on the occurrence and use levels of this specific food enzyme, is likely to have led to an overestimation of the exposure.

The exclusion of two food manufacturing processes from the exposure assessment was based on > 99% of TOS removal during these processes and is not expected to have an impact on the overall estimate derived.

3.6. Margin of exposure

A comparison of the NOAEL (1,735 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.039–0.671 mg TOS/kg bw per day at the mean and from 0.127 to 1.238 mg TOS/kg bw per day at the 95th percentile, resulted in a margin of exposure of at least 1,401.

4. Conclusions

Based on the data provided, the removal of TOS during the production of sugar syrups and in distilled alcohol production, and the derived margin of exposure for the remaining four food manufacturing processes, the Panel concluded that the food enzyme glucan 1,4-α-glucosidase produced with the non-genetically modified *R. delemar* strain CU634-1775 does not give rise to safety concerns under the intended conditions of use.

5. Documentation provided to EFSA

1) Application for the Authorisation of Glucoamylase from *Rhizopus oryzae* Strain CU634-1775. March 2015. Submitted by Shin Nihon Chemical Co., Ltd.
2) Additional data January 2021. Submitted by Shin Nihon Chemical Co., Ltd.
3) Additional data 2 March 2021. Submitted by Shin Nihon Chemical Co., Ltd.
4) Additional data 30 March 2021. Submitted by Shin Nihon Chemical Co., Ltd.

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Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
OECD	Organisation for Economic Cooperation and Development
TOS	total organic solids
WHO	World Health Organization

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

Information provided in this appendix is shown in an excel file (downloadable at https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2023.7841#support-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, 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).