

ADOPTED: 2 July 2019

doi: 10.2903/j.efsa.2019.5767

## Safety evaluation of the food enzyme chitinase from *Streptomyces violaceoruber* (strain pChi)

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Vittorio Silano, José Manuel Barat Baviera, Claudia Bolognesi, Beat Johannes Brüscheiler, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn, Boet Glandorf, Lieve Herman, Jaime Aguilera, Yi Liu and Andrew Chesson

### Abstract

The food enzyme, a chitinase (EC 3.2.1.14), is produced with the genetically modified *Streptomyces violaceoruber* strain pChi by Nagase. No information was provided regarding the presence of antimicrobial resistance genes in the production strain, other than that used in the genetic modification. The chitinase is intended to be used in baking processes. Based on the maximum use levels recommended, dietary exposure to the food enzyme–total organic solids (TOS) was estimated on the basis of individual data from the EFSA Comprehensive European Food Consumption Database. The exposure estimate is up to 0.829 mg TOS/kg body weight per day in European populations. Genotoxicity tests did not raise a safety concern. The systemic toxicity was assessed by means of a repeated dose 90-days oral toxicity study in rats. The Panel identified a no observed adverse effect level at the highest dose tested of 791 mg TOS/kg body weight, which, compared with the dietary exposure, results in margin of exposure of at least 1,171. Similarity of the amino acid sequence to those of known allergens was searched and no matches were found. The Panel considered that there are no indications for food allergic reactions to this chitinase. Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme chitinase produced with the genetically modified *S. violaceoruber* strain pChi does not give rise to safety concerns arising from the toxicological studies and the production process under the intended conditions of use. The CEP Panel was unable to conclude on the absence of viable cells and DNA from the genetically modified production strain in the food enzyme, for which uncertainty remains on the possible presence of gene(s) conferring antimicrobial resistance.

© 2019 European Food Safety Authority. *EFSA Journal* published by John Wiley and Sons Ltd on behalf of European Food Safety Authority.

**Keywords:** food enzyme, chitinase, EC 3.2.1.14, *Streptomyces violaceoruber*, pChi, genetically modified microorganism

**Requestor:** European Commission

**Question number:** EFSA-Q-2015-00621

**Correspondence:** [fip@efsa.europa.eu](mailto:fip@efsa.europa.eu)

**Panel members:** José Manuel Barat Baviera, Claudia Bolognesi, Beat Johannes Brüscheweiler, Andrew Chesson, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi, Alicja Mortensen, Gilles Rivière, Vittorio Silano, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis and Holger Zorn.

**Note:** 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 CEP Panel wishes to acknowledge all European competent institutions, Member State bodies and other organisations that provided data for this scientific output.

**Suggested citation:** EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids), Silano V, Barat Baviera JM, Bolognesi C, Brüscheweiler BJ, Cocconcelli PS, Crebelli R, Gott DM, Grob K, Lampi E, Mortensen A, Rivière G, Steffensen I-L, Tlustos C, Van Loveren H, Vernis L, Zorn H, Glandorf B, Herman L, Aguilera J, Liu Y and Chesson A, 2019. Scientific Opinion on the safety evaluation of the food enzyme chitinase from *Streptomyces violaceoruber* (strain pChi). EFSA Journal 2019;17(7):5767, 15 pp. <https://doi.org/10.2903/j.efsa.2019.5767>

**ISSN:** 1831-4732

© 2019 European Food Safety Authority. *EFSA Journal* published by John Wiley and Sons Ltd on behalf of European Food Safety Authority.

This is an open access article under the terms of the [Creative Commons Attribution-NoDerivs](https://creativecommons.org/licenses/by-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited and no modifications or adaptations are made.



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



## Table of contents

Abstract.....	1
1. Introduction.....	4
1.1. Background and Terms of Reference as provided by the requestor.....	4
1.1.1. Background as provided by the European Commission.....	4
1.1.2. Terms of Reference.....	5
1.2. Interpretation of the Terms of Reference.....	5
2. Data and Methodologies.....	5
2.1. Data.....	5
2.2. Methodologies.....	5
3. Assessment.....	5
3.1. Source of the food enzyme.....	5
3.1.1. Characteristics of the recipient microorganism.....	6
3.1.2. Characteristics of introduced sequences.....	6
3.1.3. Description of the genetic modification process.....	6
3.1.4. Safety aspects of the genetic modification.....	6
3.2. Production of the food enzyme.....	6
3.3. Characteristics of the food enzyme.....	7
3.3.1. Properties of the food enzyme.....	7
3.3.2. Chemical parameters.....	7
3.3.3. Purity.....	7
3.3.4. Viable cells and DNA of the production strain.....	8
3.4. Toxicological data.....	8
3.4.1. Genotoxicity.....	8
3.4.1.1. Bacterial reverse mutation test.....	8
3.4.1.2. <i>In vitro</i> mammalian chromosomal aberration test.....	8
3.4.2. Repeated dose 90-day oral toxicity study in rodents.....	9
3.4.3. Allergenicity.....	10
3.5. Dietary exposure.....	10
3.5.1. Intended use of the food enzyme.....	10
3.5.2. Dietary exposure estimation.....	10
3.5.3. Uncertainty analysis.....	11
3.6. Margin of exposure.....	11
4. Conclusions.....	11
Documentation provided to EFSA.....	12
References.....	12
Abbreviations.....	13
Appendix A – Dietary exposure estimates to the food enzyme–TOS in details.....	14
Appendix B – Population groups considered for the exposure assessment.....	15

## 1. Introduction

Article 3 of the Regulation (EC) No. 1332/2008<sup>1</sup> 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<sup>2</sup> 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, 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 on food enzymes.

Five applications have been introduced by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) for the authorisation of the food enzyme consisting of Protease, Leucyl aminopeptidase, Oryzin and Aspergillopepsin I from *Aspergillus oryzae* and the companies "BENEO-Palatinit GmbH" for the authorisation of the food enzyme Isomaltulose synthase from *Protaminobacter rubrum* (strain Z12A), "Nagase (Europa) GmbH" for the authorisation of the food enzyme Chitinase from a genetically modified strain of *Streptomyces violaceoruber* (strain pChi), "Clasado Ingredients Ltd." for the authorisation of the food enzyme Beta-galactosidase from a genetically modified strain of *Escherichia coli* (strain BglA MCB3) and "Meiji Seika Pharma Co., Ltd" for the authorisation of the food enzyme consisting of Aspergillopepsin I and II from *Aspergillus niger var. macrosporus* (strain DBD-0406).

Following the requirements of Article 12.1 of Regulation (EC) No 234/2011<sup>3</sup> implementing Regulation (EC) No 1331/2008<sup>4</sup>, the Commission has verified that the five applications fall within the

<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> 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, p. 15–24.

<sup>4</sup> 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, p. 1–6.

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 requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Protease, Leucyl amino-peptidase, Oryzin and Aspergillopepsin I from *Aspergillus oryzae*, Isomaltulose synthase from *Protaminobacter rubrum* (strain Z12A), Chitinase from a genetically modified strain of *Streptomyces violaceoruber* (strain pChi), Beta-galactosidase from a genetically modified strain of *Escherichia coli* (strain Bg1A MCB3) and Aspergillopepsin I and II from *Aspergillus niger* var. *macrosporus* (strain DBD-0406) 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 request to carry out the safety assessment of the food enzyme Chitinase from a genetically modified strain of *Streptomyces violaceoruber* pChi.

## 2. Data and methodologies

### 2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme chitinase produced with a genetically modified *Streptomyces violaceoruber* (strain pChi).

Additional information was requested from the applicant during the assessment process on 10 December 2018 and was consequently provided (see 'Documentation provided to EFSA'). However, some of the data requested were not provided. Consequently, the Panel concluded this assessment on the basis of the available data.

### 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) as well as in the EFSA Scientific Opinion on Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018) and following the relevant existing guidance's of EFSA Scientific Committees.

The current Guidance on the submission of a dossier on food enzymes for safety evaluation (EFSA CEF Panel, 2009) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance to the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016).

## 3. Assessment

IUBMB nomenclature:	Chitinase
Systematic name:	(1→4)-2-acetamido-2-deoxy-β-D-glucan glycanohydrolase
Synonyms:	chitodextrinase; 1,4-β-poly-N-acetylglucosaminidase; poly-β-glucosaminidase; beta-1,4-poly-N-acetyl glucosaminidase; poly[1,4-(N-acetyl-β-D-glucosaminide)] glycanohydrolase
IUBMB No:	EC 3.2.1.14
CAS No:	9001-06-3
EINECS No:	232-578-7

The chitinase catalyses the hydrolysis of N-acetyl-β-D-glucosaminide (1→4)-β-linkages in chitin and chitodextrins to form chitin oligosaccharides. It is intended to be used in baking processes.<sup>5</sup>

### 3.1. Source of the food enzyme

The chitinase production strain *Streptomyces violaceoruber* pChi is deposited in the collection of the Japanese Biological Resource Center (NBRC) under the deposit number [REDACTED]<sup>6</sup>

<sup>5</sup> See annex for list of food categories.

<sup>6</sup> Technical dossier/Annex 3.3. Att1.

### 3.1.1. Characteristics of the recipient microorganism

The recipient microorganism is the strain *Streptomyces* [REDACTED]

[REDACTED] Therefore, the strain is considered sufficiently identified. *S. violaceoruber* is known to produce secondary metabolites with antimicrobial activity and antimicrobial resistance cannot be excluded for strains of the species. No information was provided on possible antimicrobial resistances of the recipient strain.

### 3.1.2. Characteristics of introduced sequences

[REDACTED]<sup>8</sup> [REDACTED]

### 3.1.3. Description of the genetic modification process

[REDACTED]

### 3.1.4. Safety aspects of the genetic modification

[REDACTED]

## 3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No. 852/2004,<sup>9</sup> 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 submerged, batch/fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the solid biomass is removed from the fermentation broth by filtration leaving a supernatant containing the food enzyme. The filtrate containing the enzyme is then further purified and concentrated, including an ultrafiltration step in which enzyme protein is retained while most of the low molecular weight material passes the filtration membrane and is discarded. Finally, the food enzyme is dried. 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 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.

<sup>7</sup> Technical dossier/Annex 3.3. Att7.

<sup>8</sup> Technical dossier/Annex 3.3. Att4.

<sup>9</sup> 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.

### 3.3. Characteristics of the food enzyme

#### 3.3.1. Properties of the food enzyme

The mature chitinase under assessment is a single polypeptide of [REDACTED] amino acids (excluding the signal peptide), with a molecular mass of 60.6 kDa, calculated from the amino acid sequence. The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 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 60 kDa.<sup>10</sup> Minor peptidase and lipase side activities have been reported by the applicant.<sup>11</sup>

The determination of chitinase activity is based on the hydrolysis of the 4-nitrophenyl *N,N*-diacetyl- $\beta$ -D-chitobioside and is expressed in chitinase Units/g (U/g). The analytical principle is based on the release of 4-nitrophenol, which is measured by spectrophotometry at 400 nm (reaction conditions: 37°C, pH 5.5). One unit of chitinase activity (U)<sup>12</sup> is the amount of enzyme that generates 1  $\mu$ mol of 4-nitrophenol per minute.<sup>13</sup>

The food enzyme has a temperature optimum in the range of 50–60°C (at pH 5.5) and a pH optimum between 5.0 and 7.0 (at 37°C). Thermostability was tested after a pre-incubation of the food enzyme for 30 min at different temperatures. Under the conditions (pH 5.5) of the applied temperature stability assay, chitinase activity is completely inactivated above 70°C.

#### 3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for six food enzyme batches, five batches used for commercialisation<sup>14</sup> and one batch produced for the toxicological tests (Table 1). The average total organic solids (TOS) of the five food enzyme batches for commercialisation was 94.0% (range 91.0–95.0%) (Table 1). The mean enzyme activity/TOS ratio of the five food enzyme batches for commercialisation is 150.3 U/mg TOS.

**Table 1:** Compositional data of five batches of the food enzyme and the batch used for toxicological studies

Parameter	Units	Batches		
		1–5		6 <sup>(a)</sup>
		Mean	Minimum–Maximum	
Chitinase activity	U/g batch <sup>(b)</sup>	141.4	116.7–164.0	190.0
Ash	%	1.7	1.6–1.8	1.1
Water	%	4.2	3.3–7.1	1.8
Total organic solids (TOS) <sup>(c)</sup>	%	94.0	91.0–95.0	97.1
Chitinase Activity/TOS	U/g TOS	150.3	123.1–160.8	195.7

(a): Batch used for the toxicological studies.

(b): UNIT: Chitinase Unit (see Section 3.3.1).

(c): TOS calculated as 100% - % water - % ash

#### 3.3.3. Purity

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

[REDACTED] 15

<sup>10</sup> Technical dossier/Additional information June 2019/Annex R5.

<sup>11</sup> Technical dossier/Annex 2.2.

<sup>12</sup> LoQ (Limit of Quantitation): 0.005U/g.

<sup>13</sup> Technical dossier/Annex 2.3.

<sup>14</sup> Technical dossier/Additional information June 2019/Annex R1,3.

<sup>15</sup> Technical dossier/Annex 2.1. LoDs: As = 1 mg/kg; Cd = 0.01 mg/kg; Hg = 0.01 mg/kg.

The food enzyme preparation complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *E. coli* and *Salmonella* species are absent in 25 g of sample and total coliforms are not more than 30 colony forming units (CFU) per gram. [REDACTED]

[REDACTED]<sup>16</sup> No antimicrobial activity was detected in any of the tested batches (FAO/WHO, 2006).<sup>16</sup>

[REDACTED]<sup>17</sup>

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

### 3.3.4. Viable cells and DNA of the production strain

The presence of viable cells of the production strain in the food enzyme concentrate was investigated in [REDACTED]

[REDACTED] the Panel considers the amount of food enzyme tested inadequate and, although requested, not according to the indicated guidance (EFSA FEEDAP Panel, 2018). Consequently, the Panel was unable to reach a conclusion on the absence of viable cells of the production strain in the food enzyme.

[REDACTED]

[REDACTED]<sup>18</sup> However, the methodology used did not follow the indicated guidance (EFSA FEEDAP Panel, 2018) as requested from the applicant.<sup>19</sup> Consequently, the Panel was unable to reach a conclusion on the absence of DNA from the production strain in the food enzyme.

## 3.4. Toxicological data

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 has been provided. The batch 6 (Table 1) used in these studies corresponds to the enzyme concentrate used to prepare the commercial food enzyme. It shows a similar protein pattern and chemical purity as the batches used for commercialisation, and thus is considered suitable as a test item.

### 3.4.1. Genotoxicity

#### 3.4.1.1. Bacterial reverse mutation test

The bacterial reverse mutation assay (Ames test) was performed according to OECD Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP)<sup>20</sup> in four strains of *Salmonella* Typhimurium (TA100, TA1535, TA98, TA1537) and *E. coli* strain WP2 *uvrA* in the presence or absence of metabolic activation system (Aroclor 1254-induced rat liver; S9-mix), applying the pre-incubation method using different concentrations (313, 625, 1,250, 2,500 and 5,000 µg/plate of the p-Chi chitinase bulk powder, corresponding to 304, 607, 1,214, 2,428 and 4,855 µg TOS/plate). Two experiments were performed, in triplicate. Upon treatment with the food enzyme no growth inhibition and no increase in revertant colony numbers was observed at any concentration in any strain. Therefore, the Panel concluded that the food enzyme chitinase did not induce gene mutations in the bacterial reverse mutation assay under the test conditions employed for this study.

#### 3.4.1.2. *In vitro* mammalian chromosomal aberration test

The *in vitro* chromosomal aberrations test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP.<sup>21</sup> Chinese hamster lung (CHL/IU) fibroblast cells were treated with the p-Chi chitinase bulk powder of *S. violaceoruber* (strain pChi), both in the presence and absence of metabolic activation (S-9 mix). Two experiments were performed in duplicate cultures. In the presence of S9-mix, the cultured cells were exposed for 6 followed by 18 h recovery (6 + 18 h) at concentrations of 500, 1,000, 1,500 and 2,000 µg food enzyme/mL, corresponding to 486, 971, 1,457

<sup>16</sup> Technical dossier/Annex 2.1.

<sup>17</sup> Technical dossier/Annex 2.1. LoD: 1 µg/Kg.

<sup>18</sup> Technical dossier/Additional information June 2019/Annex R10.

<sup>19</sup> Request for additional information 10 December 2018.

<sup>20</sup> Technical dossier/Annex 7.1.2.

<sup>21</sup> Technical dossier/Annex 7.1.3.



and 1,942 µg TOS/mL). In the absence of S9-mix, the cells were exposed 6 + 18 h (short-term treatment) at concentrations of 1,250, 2,500 and 5,000 µg food enzyme/mL, corresponding to 1,214, 2,428 and 4,855 µg TOS/mL and also continuously for 24 + 0 h and 48 + 0 h at concentrations of 156, 313, 625 and 1,250 µg food enzyme/mL, corresponding to 151, 304, 607 and 1,214 µg TOS/mL). The percentage of cells with structural or numerical aberrations in the chitinase treated groups was not significantly increased in comparison with the solvent control at any concentration level in any experimental condition.

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

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

The repeated dose 90-day oral toxicity study in rats was performed in accordance with OECD Test Guideline 408 (OECD, 1998) and following GLP.<sup>22</sup> Groups of 10 male and 10 female Sprague–Dawley (CrI:CD(SD)) rats were treated once daily by gavage for 90 days with 40, 200 and 1,000 mg/kg body weight (bw) per day of the food enzyme p-Chi chitinase bulk powder corresponding to 39, 194, and 971 mg TOS/kg bw per day. Controls received the vehicle (sterile water).

No mortality was observed.

A statistically significantly higher food consumption than in the control group was recorded in low-dose males on day 49 of administration. As this was an isolated finding without dose–response relationship, it was not considered toxicologically relevant.

Water intake measured only on the day before urinalysis (ml/animal) of the high-dose males and females was 33% and 25% higher than in the control group and the difference reached a statistical significance for females. The Panel considered the higher water intake as a not adverse but treatment-related effect, possibly due to a higher intake of TOS.

In the open field observation, a statistically significantly lower value than in the control group was recorded in the rearing count in low-dose males in week 13. Motor activity in high-dose males was statistically significantly higher compared with the control group at 50–60 min after start. The Panel considered these findings to be of no toxicological relevance as they occurred without dose dependency and were limited to one sex.

Haematological examination revealed several statistically significant differences from controls. These were an increased relative lymphocyte count and a decreased relative neutrophil count in high-dose females, increased absolute and relative counts of large unstained cells in low- and mid-dose females, and an increase in relative eosinophil count in high-dose males. Although no symptoms of inflammation were reported in the clinical phase of the study, nor were they found in post-mortem macroscopic and microscopic examinations (see below), the relation to treatment could not be ruled out. The Panel considered these changes not to be adverse.

Among clinical chemistry parameters, statistically significantly lower values were recorded for calcium concentration in high-dose females and for alanine aminotransferase (ALT) activity in mid-dose males and high-dose females as compared with controls. The calcium concentration of high-dose females was not decreased in a dose-dependent manner and it was within the range of the relevant historical control values from the laboratory. Although the decrease in alkaline phosphatase (ALP) appeared in females to be dose-related, in the light of no changes in the absolute or relative liver weights or in morphology of this organ, this finding was considered not of toxicological significance.

The urinalysis showed a tendency toward low pH in high-dose males and females and a statistically significantly lower osmotic pressure in mid-dose females. These changes were not regarded as toxicologically significant by the Panel because even at the highest dose tested urine pH values were in the normal physiological range of rats, and the change in urine osmotic pressure was an isolated, not dose-related finding.

At necropsy, no test article-related macroscopic changes were recorded in males or females. Histopathological examination revealed hyperplasia of the squamous epithelial cells, mainly in limiting ridge of the forestomach, in three high-dose males. This finding was considered by the Panel as treatment-related but of no significance for human risk assessment, as this change represented a reaction to a repeated bolus administration of a test article at high concentrations to which consumers would not be exposed.

<sup>22</sup> Technical dossier/Annex 7.1.4.

Overall, the Panel identified a no observed adverse effect level (NOAEL) of 971 mg TOS/kg bw per day, the highest dose tested.

### 3.4.3. Allergenicity

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

The potential allergenicity of the chitinase produced with the genetically modified *S. violaceoruber* strain pChi was assessed by comparing its amino acid sequence with those of known allergens according to the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2017). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.

No food allergic reactions to this chitinase have been reported in the literature.

Several cases of respiratory allergy following occupational inhalation of aerosols containing chitinase have been reported (Martel et al., 2010; Patel and Goyal, 2017). However, several studies have shown that adults with occupational asthma to enzymes can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Brisman, 2002; Poulsen, 2004; Armentia et al., 2009).

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

## 3.5. Dietary exposure

### 3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used in baking process at a recommended use level of up to 69.7 mg TOS/kg wheat flour.

In baking processing, the chitinase food enzyme preparation is added to the raw materials during the preparation of the dough. According to the applicant, the food enzyme acts on yeasts cell walls promoting the separation of yeast cells and better distribution within the dough. This in turn can improve dough quality.

The food enzyme remains in the dough. Based on data provided on thermostability (see Section 3.3.1), it is expected that the chitinase is inactivated during baking processes.

### 3.5.2. Dietary exposure estimation

Chronic exposure was calculated by combining the maximum recommended use level provided by the applicant (see Section 3.5.1) with the relevant FoodEx categories (Annex B in EFSA CEF Panel, 2016), based on individual consumption data. Exposure from individual FoodEx categories was subsequently summed up, averaged over the total survey period 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 average and 95th percentile exposures were calculated per survey for the total population and per age class. Surveys with only one 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 35 different dietary surveys (covering infants, toddlers, children, adolescents, adults and the elderly), carried out in 22 European countries (Appendix B).

**Table 2:** 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)	0.013–0.194 (10)	0.147–0.417 (14)	0.167–0.403 (19)	0.091–0.257 (18)	0.069–0.160 (19)	0.068–0.142 (18)
Min–max 95th percentile (number of surveys)	0.076–0.829 (8)	0.367–0.710 (12)	0.328–0.756 (19)	0.204–0.523 (17)	0.151–0.313 (19)	0.136–0.248 (18)

TOS: total organic solid.

### 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 3.

**Table 3:** Qualitative evaluation of the influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction of impact
<b>Model input data</b>	
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	+/-
<b>Model assumptions and factors</b>	
FoodEx categories included in the exposure assessment were assumed to always contain the food enzyme-TOS	+
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	+/-

+: uncertainty with potential to cause overestimation of exposure; -: uncertainty with potential to cause underestimation of exposure.

TOS: total organic solid.

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 a considerable overestimation of the exposure.

### 3.6. Margin of exposure

A comparison of the NOAEL (971 mg TOS/kg bw per day) from the 90-day rat study with the derived exposure estimates of 0.013–0.417 mg TOS/kg bw per day at the mean and from 0.076–0.829 mg TOS/kg bw per day at the 95th percentile, resulted in margin of exposure of at least 1,171.

## 4. Conclusions

Based on the data provided and the derived margin of exposure, the Panel concluded that the food enzyme chitinase produced with the genetically modified *S. violaceoruber* strain pChi does not give rise to safety concerns arising from the toxicological studies and the production process under the intended conditions of use.

The CEP Panel was unable to conclude on the absence of viable cells and DNA from the genetically modified production strain in the food enzyme, for which uncertainty remains on the possible presence of gene(s) conferring antimicrobial resistance.

## Documentation provided to EFSA

- 1) Request for the authorization of a chitinase preparation from *Streptomyces violaceoruber* pChi for use as a food processing aid. October 2015. Submitted by Nagase (Europa) GmbH
- 2) Additional information. June 2019. Submitted by Regal B.V.
- 3) Summary report on GMM part for chitinase produced by *Streptomyces violaceoruber* strain pChi, EFSA-Q-2015-00621. 2017. Delivered by Technical University of Denmark.

## References

- Armentia A, Díaz-Perales A, Castrodeza J, Dueñas-Laita A, Palacin A and Fernández S, 2009. Why can patients with baker's asthma tolerate wheat flour ingestion? Is wheat pollen allergy relevant? *Allergologia et Immunopathologia*, 37, 203–204.
- Brisman J, 2002. Baker's asthma. *Occupational and Environmental Medicine*, 59, 498–502; quiz 502, 426.
- Cruz-Morales P, Vigenboom E, Iruegas-Bocardo F, Girard G, Yànez-Guerra LA, Ramos-Aboite HE, Pernodet JL, Anné J, van Wezel P and Barona-Gómez F, 2013. The genome sequence of *Streptomyces lividans* 66 reveals a novel tRNA-dependent peptide biosynthetic system within a metal-related genomic island. *Genome Biology and Evolution*, 5, 1165–1175.
- EFSA (European Food Safety Authority), 2006. Opinion of the Scientific Committee related to uncertainties in dietary exposure assessment. *EFSA Journal* 2006;5(1):438, 54 pp. <https://doi.org/10.2903/j.efsa.2007.438>
- EFSA (European Food Safety Authority), 2009. Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessments carried out by EFSA. Part 2: general principles. *EFSA Journal* 2009;7(5):1051, 22 pp. <https://doi.org/10.2903/j.efsa.2009.1051>
- EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in exposure assessment. *EFSA Journal* 2011;9(3):2097, 34 pp. <https://doi.org/10.2903/j.efsa.2011.2097>
- EFSA CEF Panel (EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids), 2009. Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the submission of a dossier on food enzymes. *EFSA Journal* 2009;7(8):1305, 26 pp. <https://doi.org/10.2903/j.efsa.2009.1305>
- EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016. Exposure assessment of food enzymes. *EFSA Journal* 2016;14(11):4581, 9 pp. <https://doi.org/10.2903/j.efsa.2016.4581> and Annex B – Process-specific technical data used in exposure assessment of food enzymes (Available online: [https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2016.4581&file=efs24581-sup-0001-Annex\\_B.pdf](https://efsa.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.2903%2Fj.efsa.2016.4581&file=efs24581-sup-0001-Annex_B.pdf))
- EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal* 2018;16(3):5206, 24 pp. <https://doi.org/10.2903/j.efsa.2018.5206>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Birch AN, Casacuberta J, De Schrijver A, Galak MA, Guerche P, Jones H, Manachini B, Messean A, Nielsen EE, Nogue F, Robaglia C, Rostoks N, Sweet J, Tebbe C, Visioli F, Wal J-M, Eigenmann P, Epstein M, Hoffmann-Sommergruber K, Koning F, Lovik M, Mills C, Moreno FJ, van Loveren H, Selb R and Fernandez Dumont A, 2017. Guidance on allergenicity assessment of genetically modified plants. *EFSA Journal* 2017;15(5):4862, 49 pp. <https://doi.org/10.2903/j.efsa.2017.4862>
- Euzéby JP, 1997. List of bacterial names with standing in nomenclature: a folder available on the internet. *International Journal of Systematic Bacteriology*, 47, 590–592.
- FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), 2006. General specifications and considerations for enzyme preparations used in food processing in Compendium of food additive specifications. 67th meeting. FAO JECFA Monographs, 3, 63–67 Available online: <http://www.fao.org/3/a-a0675e.pdf>
- Lewis RA, Laing E, Allenby N, Bucca G, Brenner V, Harrison M, Kierzek AM and Smith CP, 2010. Metabolic and evolutionary insights into the closely-related species *Streptomyces coelicolor* and *Streptomyces lividans* deduced from high-resolution comparative genomic hybridization. *BMC Genomics*, 11, 682.
- Martel C, Nielsen GD, Mari A, Licth TR and Poulsen LK, 2010. Bibliographic review on the potential of microorganisms, microbial products and enzymes to induce respiratory sensitization. *EFSA Supporting Publication* 2010; 7(9):EN-75, 95 pp. <https://doi.org/10.2903/sp.efsa.2010.en-75>

- OECD (Organisation for Economic Co-Operation and Development), 1997a. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 471: Bacterial reverse mutation test. 21 July 1997. 11 pp. Available online: [http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test\\_9789264071247-en;jsessionid=9zfgzu35paaq.x-oecd-live-01](http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en;jsessionid=9zfgzu35paaq.x-oecd-live-01)
- OECD (Organisation for Economic Co-Operation and Development), 1997b. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 473: *In vitro* mammalian chromosomal aberration test. 21 July 1997. 10 pp. Available online: [https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test\\_9789264071261-en](https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosome-aberration-test_9789264071261-en)
- OECD (Organisation for Economic Co-Operation and Development), 1998. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 408: Repeated dose 90-day oral toxicity study in rodents. 21 September 1998. 10 pp. Available online: [http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents\\_9789264070707-en](http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en)
- Patel S and Goyal A, 2017. Chitin and chitinase: Role in pathogenicity, allergenicity and health. *International Journal of Biological Macromolecules*, 97, 331–338.
- Poulsen LK, 2004. Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel food. *Molecular Nutrition & Food Research*, 48, 413–423.

## Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
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
CFU	colony forming units
CHL	Chinese hamster lung
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organization of the United Nations
GLP	Good Laboratory Practice
GMM	genetically modified microorganism
GMO	EFSA Panel on Genetically Modified Organisms
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LoD	limit of detection
LoQ	limit of quantitation
NOAEL	no observed adverse effect level
NBRC	Japanese Biological Resource Center
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
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 <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2019.5767>).

The file contains two sheets, corresponding to two tables.

Table 1: Mean 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 one day
Infants	From 12 weeks on up to and including 11 months of age	Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, United Kingdom
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, United Kingdom
Children <sup>(a)</sup>	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Netherlands, Portugal, Spain, Sweden, United Kingdom
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom
The elderly <sup>(a)</sup>	From 65 years of age and older	Austria, Belgium, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom

(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).