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Safety evaluation of the food enzyme cyclomaltodextrin glucanotransferase from the non-genetically modified *Anoxybacillus caldiproteolyticus* strain TCM3-539

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Claude Lambré, José Manuel Barat Baviera, Claudia Bolognesi, Pier Sandro Cocconcelli, Riccardo Crebelli, David Michael Gott, Konrad Grob, Evgenia Lampi, Marcel Mengelers, Alicja Mortensen, Gilles Rivière, Inger-Lise Steffensen, Christina Tlustos, Henk Van Loveren, Laurence Vernis, Holger Zorn, Jaime Aguilera, Magdalena Andryszkiewicz, Yi Liu and Andrew Chesson

Abstract

The food enzyme cyclomaltodextrin glucanotransferase ((1-4)-α-D-glucan:(1-4)-α-D-glucan 4-α-D-[(1-4)-α-D-glucano]-transferase; EC 2.4.1.19) is produced with the non-genetically modified bacteria *Anoxybacillus caldiproteolyticus* strain TCM3-539 by Hayashibara Co., Ltd. It is free from viable cells of the production strain. The food enzyme is intended to be used for the manufacture of glucosyl hesperidin and ascorbic acid 2-glucoside. Since residual amounts of total organic solids are removed by filtration, adsorption, chromatography and crystallisation, dietary exposure estimation was considered not necessary. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and one 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 the food enzyme does not give rise to safety concerns under the intended conditions of use.

Keywords: food enzyme, cyclomaltodextrin glucanotransferase, ((1-4)-α-D-glucan:(1-4)-α-D-glucan 4-α-D-[(1-4)-α-D-glucano]-transferase, EC 2.4.1.19, cyclodextrin glycosyltransferase, *Anoxybacillus caldiproteolyticus*

Requestor: European Commission

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Correspondence: fip@efsa.europa.eu

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

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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:

- 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, 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 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.

An application has been introduced by Hayashibara Co., Ltd, for the authorisation of the food enzyme: Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*.

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 application falls within the scope of the food enzyme Regulation and contains all the elements required under Chapter II of that Regulation.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessment on the following food enzyme: Cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus* in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

[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.

1.1.3. 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 cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*. Recent data identified the production microorganism as *Anoxybacillus caldiproteolyticus* strain TCM3-539 (Section 3.1). Therefore, this name will be used in this opinion instead of *Geobacillus stearothermophilus*.

2. Data and Methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme cyclomaltodextrin glucanotransferase from *Geobacillus stearothermophilus*.

Additional information was requested from the applicant during the assessment process on 12 January 2021 and 12 May 2022 and subsequently 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 existing guidance documents of EFSA Scientific Committee.

The current '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.

3. Assessment

IUBMB nomenclature	Cyclomaltodextrin glucanotransferase
Systematic name	(1-4)-α-D-glucan 4-α-D-[(1-4)-α-D-glucano]-transferase (cycling)
Synonyms	Cyclodextrin glycosyltransferase, α-cyclodextrin glucanotransferase,
IUBMB No	EC 2.4.1.19
CAS No	9030-09-5
EINECS No	618-522-8

Cyclomaltodextrin glucanotransferases typically catalyse the transglycosylation of glucans by formation of a (1–4)-α-D-glucosidic bond, resulting in the generation of α-, β- and γ-cyclodextrins and transglycosylated products. The enzyme under assessment is intended to be used for the manufacture of glucosyl hesperidin (GH) and ascorbic acid 2-glucoside (AsA-G).

3.1. Source of the food enzyme

The cyclomaltodextrin glucanotransferase is produced with the non-genetically modified bacterium *Anoxybacillus caldiproteolyticus* TCM3-539 (formerly *Geobacillus stearothermophilus*), which is deposited at the National Institute of Technology and Evaluation (NITE), Biological Resource Center (Japan) with the deposit number NITE SD 00492.[4] The strain was identified as *A. caldiproteolyticus* by whole genome sequence (WGS) analysis, [redacted].

A. caldiproteolyticus TCM3-539 did not show evidence of cytotoxicity in VERO cells using lactate dehydrogenase activity as a measure of cell injury.[6] WGS analysis of the production strain [redacted] did not identify genes encoding antimicrobial resistance or virulence factors of concern.[5]

[4] Technical dossier/Additional data April 2022/Annex 1.
[5] Technical dossier/Additional data April 2022/Annex 2 and 3.
[6] Technical dossier/Additional data April 2022/Annex 3.

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004,[7] with food safety procedures based on Hazard Analysis and Critical Control Points, and in accordance with current Good Manufacturing Practice.[8]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, 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. 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 mass material passes the filtration membrane and is discarded.[9] 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.[10]

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 cyclomaltodextrin glucanotransferase is a single polypeptide chain of 680 amino acids. The molecular mass of the mature protein, calculated from the amino acid sequence, is around 75 kDa.[11] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. 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 70 kDa, consistent with the expected mass of the enzyme. The protein profile also included bands of lesser staining intensity.[12] The food enzyme was tested for protease and lipase activities; only protease activity was detected.[13] No other enzyme activities were reported.

The in-house determination of cyclomaltodextrin glucanotransferase activity is based on hydrolysis of starch (reaction conditions: pH 5.5, 40°C, 10 min). The enzyme activity is measured by quantifying the remaining starch, which is coloured with iodine and detected spectrophotometrically at 660 nm. The enzyme activity is expressed in Units (U)/g. One U is defined as the amount of enzyme that hydrolyses 15 mg of soluble starch under the conditions of the assay.[14]

The food enzyme has a temperature optimum around 75°C (pH 5.5) and a pH optimum around pH 5.5 (40°C).[15] Thermostability was tested after the incubation of the food enzyme at 60°C and 80°C (pH 5.5) for different periods. Cyclomaltodextrin glucanotransferase showed no residual activity after 10 min of incubation at 80°C.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for three batches for commercialisation (Table 1).[16] The mean total organic solids (TOS) is 4.4% and the mean enzyme activity/TOS ratio is 73.5 U/mg TOS.

[7] 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.
[8] Technical dossier/p. 41, Annex 10 and Additional data April 2022/Annex 4.
[9] Technical dossier/pp. 41–49.
[10] Technical dossier/pp. 44, 46,Annex 2 and Additional data April 2022/Annexes 5 and 6.
[11] Technical dossier/p. 31.
[12] Technical dossier/Additional data April 2022/Annex 7.
[13] Technical dossier/pp. 33–34 and Annex 6.
[14] Technical dossier/pp. 32–34 and Annex 5.
[15] Technical dossier/p. 34.
[16] Technical dossier/Additional data April 2022/Annexes 7 and 8.

Table 1: Composition of the food enzyme

Parameters	Unit	Batches		
		1	2	3
Cyclomaltodextrin glucanotransferase activity	U/g[(a)]	3,340	3,210	3,070
Protein	%	3.7	3.7	4.7
Ash	%	0.2	0.2	0.2
Water	%	95.9	95.5	94.7
Total organic solids (TOS)[(b)]	%	3.9	4.3	5.1
Cyclomaltodextrin glucanotransferase activity/TOS	U/mg TOS	85.6	74.7	60.2

(a): U/g: Cyclomaltodextrin glucanotransferase Units (see Section 3.3.1).
(b): TOS calculated as 100% - % water - % ash.

3.3.3. Purity

The lead content in the four batches 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).[17,18]

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).[19] No antimicrobial activity was detected in any of the tested batches.

The presence of aflatoxins B1, B2, G1 and G2 was examined in the three food enzyme batches and all were below the limits of detection (LoDs) of the applied analytical methods.[20,21]

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 viable cells of the production strain in the food enzyme was demonstrated [redacted].[22]

3.4. Toxicological data

The food enzyme is intended to be used in starch processing for the manufacture of GH and AsA-G. In the course of these processes, the food enzyme is removed from the final product by the applied purification steps (see Section 3.5) and, consequently, the Panel concluded that no toxicological studies other than assessment of allergenicity are needed for the assessment of this food enzyme.

3.4.1. 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 cyclomaltodextrin glucanotransferase produced with *A. caldiproteolyticus* strain TCM3-539 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. The matching allergen was Asp o 21, an α-amylase produced by *Aspergillus oryzae* known as an occupational respiratory allergen.[23]

No information is available on oral and respiratory sensitisation or elicitation reactions of this cyclomaltodextrin glucanotransferase.

[17] Technical dossier/pp. 29–30 and Annexes: 1, 11.
[18] LoDs: lead = 2 mg/kg; aflatoxins (each) = 1.0 μg/kg.
[19] Technical dossier pp. 29–30 and Annexes: 1, 11.
[20] Technical dossier/pp. 38–39 and Annex 4.
[21] Technical dossier/p. 37and Annex 3.
[22] Technical dossier/Additional data October 2022/Annex 2.
[23] Technical dossier/pp. 59–61 and Annexes 9 and 15.

α-Amylase from *A. oryzae* (Brisman and Belin, 1991; Sander et al., 1998; Brisman, 2002; Quirce et al., 2002) is known as an occupational respiratory allergen associated with baker's asthma. However, several studies have shown that adults with occupational asthma to a food enzyme (as described for α-amylase from *A. oryzae*) can ingest respiratory allergens without acquiring clinical symptoms of food allergy (Cullinan et al., 1997; Poulsen, 2004; Armentia et al., 2009). Considering the wide use of α-amylase as a food enzyme, only a low number of case reports has been described in the literature focused on allergic reactions upon oral exposure to α-amylase in individuals respiratory sensitised to α-amylase (Losada et al., 1992; Quirce et al., 1992; Baur and Czuppon, 1995; Kanny and Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004).

[redacted] and [redacted], known sources of allergens, are present in the media fed to the microorganisms. However, during the fermentation process, these products will be degraded and utilised by the microorganisms for cell growth, cell maintenance and production of enzyme protein. In addition, the microbial biomass and fermentation solids are removed. Taking into account the fermentation process and downstream processing, the Panel concluded that potentially allergenic residues of these materials employed as protein sources are not 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, 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 for the manufacture of GH and AsA-G at a recommended use level of 0.2–2,500 mg TOS/kg starch.[25]

In the manufacture of GH, the food enzyme is added to the raw materials (dextrin and the flavanone glycoside hesperidin), where the cyclodextrin glucanotransferase transfers glycosyl groups to hesperidin. The reaction products (containing GH) are treated by heat to inactivate the enzyme and purified, using different filtration methods and adsorption steps.[24]

In the manufacture of AsA-G, the food enzyme is added to the raw materials (ascorbic acid and liquefied starch) together with other enzymes, where the cyclodextrin glucanotransferase transfers glycosyl groups to ascorbic acid. The reaction products (containing AsA-G) are treated by heat to inactivate the enzymes and purified using different filtration methods, chromatography and crystallisation steps.[24]

The applicant analysed the purified GH and AsA-G products (three lots each), as well as their intermediate manufacturing samples (before purification), by enzyme-linked immune sorbent assay (ELISA), using an antibody specific to the cyclodextrin glucanotransferase.[26,27] The cyclodextrin glucanotransferase was not detected in purified GH and AsA-products, showing the removal of the enzyme protein by purification steps.[28]

3.5.2. Dietary exposure estimation

The Panel considered the enzyme protein as a proxy for the food enzyme–TOS and accepted the evidence provided as sufficient to conclude that the residual amounts of food enzyme–TOS in the final GH and AsA-G are negligible. Consequently, a dietary exposure was not calculated.

4. Conclusions

Based on the data provided, and the removal of TOS during the intended food production process, the Panel concluded that the food enzyme cyclomaltodextrin glucanotransferase produced with *A. caldiproteolyticus* strain TCM3-539 does not give rise to safety concerns under the intended conditions of use.

[24] Technical dossier/Spontaneous additional data November 2022/Annex 5a.
[25] Technical dossier/Technical dossier/p. 56.
[26] Technical dossier/Spontaneous additional data November 2022/Report on the results of TC Enzyme protein determination by ELISA_ver.2 Final check.
[27] LoD = LoQ = 1.95 ng/mL.
[28] Spontaneous additional data November 2022/ Report of result and Annexes 5b, 5c, 5d, 5 e.

5. Documentation as provided to EFSA

Application for authorisation of cyclomaltodextrin glucanotransferase preparation from *Geobacillus stearothermophilus* (Former: *Bacillus stearothermophilus*). December 2016. Submitted by Hayashibara Co., Ltd.

Additional data April 2022. Submitted by Hayashibara Co., Ltd.

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Abbreviations

AsA-G	ascorbic acid 2-glucoside
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
ELISA	enzyme-linked immune sorbent assay
FAO	Food and Agricultural Organization of the United Nations
GH	glucosyl hesperidin
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LoD	limit of detection
LoQ	limit of quantification
TOS	total organic solids
WGS	whole genome sequence
WHO	World Health Organization