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Safety evaluation of the food enzyme D-tagatose 3-epimerase from the genetically modified *Escherichia coli* strain PS-Sav-001

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Abstract

The food enzyme D-tagatose 3-epimerase (EC 5.1.3.31) is produced with the genetically modified *Escherichia coli* strain PS-Sav-001 by SAVANNA Ingredients GmbH. The genetic modifications do not give rise to safety concerns. The food enzyme is considered free from viable cells of the production organism and its DNA. The food enzyme is used while retained inside a membrane reactor to convert D-fructose into the speciality carbohydrate D-allulose (syn. D-psicose). Since residual amounts of total organic solids (TOS) are removed by the purification steps applied during the production of D-allulose, dietary exposure was not calculated and toxicological studies were not considered necessary. A search for the similarity of the amino acid sequence of the food enzyme to known allergens was made and no match 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.

Keywords: food enzyme, D-tagatose 3-epimerase, EC 5.1.3.31, L-ribulose 3-epimerase, *Escherichia coli*, genetically modified microorganism

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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.

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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 microorganisms or products thereof including a product obtained by a fermentation process using microorganisms: (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 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.

An application has been introduced by the applicant "SAVANNA Ingredients GmbH." for the authorisation of the food enzyme D-tagatose-epimerase SD16 from a genetically modified strain of *E. coli* BL21 (DE3).

Following the requirements of Article 12.1 of Commission 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: D-tagatose-epimerase SD16 from a genetically modified strain of *E. coli* BL21 (DE3) 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, 11.03.2011, pp. 15–24.

1.2. Interpretation of Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of the food enzyme D-tagatose-epimerase SD16 from a genetically modified strain of *E. Coli* BL21 (DE3). The applicant provided clarification to the name of the production strain in January 2023. Based on this, *E. coli* strain PS-Sav-001 will be used in this opinion instead of *E. coli* strain BL21 (DE3).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme D-tagatose 3-epimerase from a genetically modified *Escherichia coli* strain PS-Sav-001.

Additional information was requested from the applicant during the assessment process on 27 September 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) as well as in the 'Statement on characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) and following the relevant existing guidance of EFSA Scientific Committees.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) 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	D-tagatose 3-epimerase
Systematic name	D-tagatose 3-epimerase
Synonyms	L-ribulose 3-epimerase; ketose 3-epimerase
IUBMB No	EC 5.1.3.31
CAS No	1219591-85-1
EINECS No	807-816-3

D-Tagatose 3-epimerases catalyse the conversion of D-fructose to D-allulose (also known as D-psicose). The food enzyme is intended to be used for the production of the specialty carbohydrate D-allulose.

3.1. Source of the food enzyme

The D-tagatose 3-epimerase is produced with the genetically modified bacterium *Escherichia coli* strain PS-Sav-001, which is deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), with the deposit number [redacted][4]

The production strain was identified as *E. coli* based on whole genome sequence (WGS) data, [redacted] No acquired antimicrobial resistance (AMR) genes or potential virulence genes were identified.[5]

3.1.1. Characteristics of the parental and recipient microorganisms

The parental microorganism (*E. coli* strain BL21 (DE3)) also acts as the recipient strain. It is a non-pathogenic laboratory strain (Chart et al., 2000; Daegelen et al., 2009) whose complete genome sequence is publicly available (Jeong et al., 2009).

[4] Technical dossier/A1 GMM Dossier/A 1.10.
[5] Technical dossier/A1 GMM Dossier/A 1.01.
[6] Technical dossier/A1 GMM Dossier/A 1.02.

3.1.2. Characteristics of introduced sequences

[redacted]

3.1.3. Description of the genetic modification process

The purpose of genetic modification was to enable the production strain to synthesise the D-tagatose 3-epimerase from [redacted][7]

3.1.4. Safety aspects of the genetic modification

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

The production strain *E. coli* PS-Sav-001 differs from the recipient strain in its capacity to produce the D-tagatose 3-epimerase from [redacted] The insertion of the expression cassette was shown by WGS data.[6]

The absence of the antimicrobial resistance genes used during the genetic modification was shown by WGS analysis. WGS data also showed that [redacted][6]

No issues of concern arising from the genetic modifications were identified by the panel.

3.2. Production of the food enzyme

The food enzyme is currently manufactured in-house only in pilot scale [redacted] and according to the Food Hygiene Regulation (EC) No 852/2004[8]. A hazard analysis and critical control points (HACCP) plan is applicable only when production has been scaled-up but will be based on the experience gained from the pilot-scale operations.[9]

The production strain is grown as a pure culture using a typical industrial medium in a submerged, fed-batch fermentation system with conventional process controls in place. [redacted] After completion of the fermentation, bacterial cells are disrupted [redacted] Solids are then removed from the broth by filtration, leaving a filtrate containing the food enzyme.[10] Finally, the food enzyme is formulated [redacted] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[11]

The Panel considered that sufficient information was provided on the manufacturing process at the pilot scale.

[7] Technical dossier/A1 GMM Dossier/A 1.
[8] 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.
[9] Technical dossier/2nd submission/pg. 33, 41/A 3.2.2.2.
[10] Technical dossier/2nd submission/pg. 33-41/A 3.2.2.2.
[11] Technical dossier/2nd submission/pg. 33-41/A 3.2.2.2.01–3.2.2.2.27.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The D-tagatose 3-epimerase is a single polypeptide chain of [redacted] amino acids. The molecular mass of the mature protein, calculated from the amino acid sequence, was [redacted][12] The food enzyme was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A consistent protein pattern was observed across all batches. The gel showed a single major protein band corresponding to an apparent molecular mass of about [redacted], consistent with the expected mass of the enzyme. The protein profile also included bands of lesser staining intensity.[13] No other enzymatic activities were reported.

The in-house determination of D-tagatose 3-epimerase activity is based on epimerisation of fructose (reaction conditions: pH 7.5, 60°C, 105 min). The enzymatic activity is determined by measuring the formation of allulose by high-performance liquid chromatography (HPLC) analysis. The activity of D-tagatose 3-epimerase is expressed in U/mL. One unit is defined as the amount of enzyme that leads to the formation of 1 μmol allulose/min, under the assay conditions.[14]

The food enzyme has a temperature optimum around 70°C (pH 6.0) and a pH optimum between pH 5.8 and 8.0 (60°C). No residual activity was determined after incubation of the enzyme at 80°C for 2 h.

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme preparation were provided for three batches intended for eventual commercialisation (Table 1).[15] The mean total organic solids (TOS) of the three batches was 0.9% (g/100 mL) and the mean enzyme activity/TOS ratio was 162,512 U/mg TOS.

Table 1 Composition of the food enzyme preparation

Parameters	Unit	Batches		
		1	2	3
D-tagatose 3-epimerase activity	U/mL[(a)]	1,149	1,224	1,673
Protein	g/100 mL	1.28	1.16	1.00
Dry matter	g/100 mL	30.1	30.3	32.6
Ash	g/100 mL	0.41	0.43	0.41
Water	g/100 mL	81.92	81.57	80.17
Fructose	g/100 mL	22.85	22.94	24.93
Allulose	g/100 mL	6.05	5.93	6.54
Total organic solids (TOS)	g/100 mL	0.78	1.03	0.76
D-tagatose 3-epimerase activity/TOS	U/mg TOS	147,797	119,322	220,419

(a): U: Unit (see Section 3.3.1).

3.3.3. Purity

The lead content in the three 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). In addition, the levels of cadmium, arsenic and mercury were below the limits of quantification (LoQs) of the employed methods.[16]

The food enzyme preparation complies with the microbiological criteria (for total coliforms, *Escherichia coli* and *Salmonella*) as laid down in the general specifications for enzymes used in food processing (FAO/WHO, 2006). No antimicrobial activity was detected in any of the tested batches.[17]

[12] Technical dossier/2nd submission/pg. 15/A 3.1.2.12.
[13] Technical dossier/2nd submission/pg. 16/A 3.1.2.01 & 3.1.2.02.
[14] Technical dossier/2nd submission/pg. 22-27/A 3.1.2.13.
[15] Technical dossier/2nd submission/pg. 18, 27/A 3.1.2.07–09.
[16] LoQs: Pb, Cd, Hg = 0.01 mg/kg, each; As = 0.1 mg/kg.
[17] Technical dossier/2nd submission/pg. 19/A 3.1.2.07–09.

The [redacted] concentration in three batches of the food enzyme preparation was between 0.5 ng/mL and 1.52 ng/mL, corresponding to 5.0 mg/kg and 15.2 mg/kg enzyme preparation.[18] The Panel does not expect adverse effects related to traces amount of [redacted] in the food enzyme preparation.

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 absence of viable cells of the production strain in the food enzyme was demonstrated [redacted][19]

The absence of recombinant DNA in the food enzyme was demonstrated [redacted][20]

3.4. Toxicological data

Taking into account the intended use, that the enzyme is retained in a membrane reactor and is physically separated from the substrate the exposure to the food enzyme is considered negligible (see Section 3.5.1). Therefore, toxicological tests are not considered necessary for the assessment of this food enzyme.

Reports of a battery of toxicological tests made with D-allulose were provided by the applicant.[21] Since they were performed with the product and not with the food enzyme itself, these tests were not considered relevant for the assessment of the food enzyme and are not reported in the opinion.

3.4.1. Allergenicity

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

The potential allergenicity of the D-tagatose 3-epimerase produced with the genetically modified *Escherichia coli* strain PS-Sav-001 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, no match was found.[22]

No information is available on oral and respiratory sensitisation or elicitation reactions of this D-tagatose 3-epimerase.

The applicant conducted a literature search looking for possible allergic reactions to allulose-producing enzymes and no relevant reports were found. In addition, no allergic reactions to ingestion or respiratory exposure to epimerases have been reported in the literature.

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

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used retained inside a membrane reactor[23] to produce exclusively the specialty carbohydrate D-allulose (syn. D-psicose).[24] The typical use level is 20,000 U/kg fructose, corresponding to 50–2,000 mg TOS/kg D-fructose.[25]

[18] Technical dossier/Additional data October 2022/3.1.2.24.
[19] Technical dossier/Additional data October 2022/3.1.2.22.
[20] Technical dossier/Additional data October 2022/3.1.2.10 and 3.1.2.11.
[21] Technical dossier/Annexes-Toxicological data.
[22] Technical dossier/2nd submission/pg. 49–50.
[23] Additional data October 2022/Answer to question 13.1.
[24] Additional data October 2022/Answer to question 11.
[25] Additional data October 2022/Answer to question 12.

Due to the cut-off size of the membrane which prevents the migration of proteins and the further downstream purification process which will remove low molecular mass material, the transfer of TOS into the final product, i.e. highly purified D-allulose, is expected to be negligible. Following the epimerisation step, the reaction product (non-purified D-allulose) is subjected to a series of purification steps (a chromatographic step and decolouring for the syrup form, an additional crystallisation for the crystalline form), which are expected to eliminate any residual food enzyme from the final product D-allulose. The applicant determined the total nitrogen content and measured ash in ready-to-consume and commercially available allulose products[26] (five batches of syrups and five batches of crystalline allulose), and the results were all below the limit of detection (LoD).[27] The allulose content, measured by HPLC, was at least 65% in the syrup products and 99% in the crystalline products. The fructose content was about 0.6% in the syrup products and less than 0.1% in the crystalline products.

The D-tagatose 3-epimerase gene was not detected by PCR in five batches of D-allulose syrups.[28] No hazard was identified during the manufacturing process of the food enzyme (see Section 3.2).

3.5.2. Dietary exposure estimation

The Panel accepted the evidence provided as sufficient to conclude that the presence of residual amounts of TOS after processing of D-allulose is negligible. Consequently, a dietary exposure was not calculated.

4. Conclusions

Based on the data provided and the removal of TOS during the purification steps applied during the production of D-allulose, the Panel concluded that the food enzyme D-tagatose 3-epimerase produced with the genetically modified *Escherichia coli* strain PS-Sav-001 does not give rise to safety concerns under the intended conditions of use.

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

5. Documentation as provided to EFSA

1) Dossier "Application for the authorization of D-tagatose-epimerase SD16 as food enzyme". February 2021. Submitted by SAVANNA Ingredients GmbH. The dossier was updated on 6 October 2022.
2) Additional information. October 2022. Submitted by SAVANNA Ingredients GmbH.

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[26] Additional data October 2022/Answer to question 13.3.
[27] Technical dossier/Annexes-dietary exposure/S01 to S10. LoD for nitrogen = 0.1 g/100 g, LoD for ash = 0.1 g/100 g.
[28] Technical dossier/Annexes-dietary exposure/S11.

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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
DSMZ	Leibniz-Institut – German Collection of Microorganisms and Cell Cultures
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation of the United Nations
GLP	Good Laboratory Practice
GMM	genetically modified microorganism
GMO	genetically modified organism
HACCP	Hazard analysis and critical control points
HPLC	high performance liquid chromatography
[redacted]	
IUBMB	International Union of Biochemistry and Molecular Biology
kDa	kilodalton
LoD	limit of detection
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TOS	total organic solids
WGS	whole genome sequence
WHO	World Health Organization