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D-ALLULOSE 3-EPIMERASE ENZYME PREPARATION FROM *ARTHROBACTER GLOBIFORMIS* EXPRESSED IN A GENETICALLY MODIFIED STRAIN OF *ESCHERISCHIA COLI*

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the D-allulose 3-epimerase enzyme preparation from *Arthrobacter globiformis* expressed in *Escherischia coli*. The information for evaluation was submitted to JECFA in a dossier dated 15 February 2020. This document also discusses published information relevant to D-allulose 3-epimerase, the *E. coli* production organism, and the *Arthrobacter globiformis* organism that is the source for the D-allulose 3-epimerase gene. This document uses the expression "D-allulose 3-epimerase" to refer to the enzyme and its amino acid sequence, and the expression "D-allulose 3-epimerase preparation" to refer to the product formulated for commercial use.

D-Allulose 3-epimerase is an enzyme that catalyses the reversible epimerization of keto-sugars at the C3 position, primarily in allulose (also known as psicose). The 3-epimerase enzyme preparation is used as a processing aid to convert D-fructose to D-allulose, a rare ketohexose (e.g. from corn syrup) used as a sweetener in foods.

D-Allulose epimerase is manufactured by a controlled aerobic batch fermentation of a pure culture of a genetically modified strain of *E. coli* containing the D-allulose 3-epimerase gene from *A. globiformis*. *Escherichia coli* K12 has been safely used in the production of chymosin enzyme, otherwise known as rennin, used in cheese production, for many years without known side effects (Flamm, 1991). No evidence or indications of pathogenicity of *E. coli* K-12 are known (Olempska-Beer et al., 2006). The gene encoding the D-allulose 3-epimerase was isolated from *A. globiformis* strain M30, which was deposited in the National Institute of Technology and Evaluation (NITE) in Japan under accession number P-1111 (Yoshihara et al., 2017). The production strain *E. coli* K-12 W3110 (pWKLP) was constructed to introduce the *A. globiformis* gene encoding D-allulose 3-epimerase into the host strain, *E. coli* K-12 W3310. After the main fermentation is stopped by bacteriolysis (heat and lysozyme for 18h), the enzyme is extracted from the cell material. This is followed by a series of filtration steps in which the liquid enzyme is concentrated and purified. The liquid enzyme is formulated into the commercial D-allulose 3-epimerase preparation by the addition of water and sugar alcohols; the powdered product is prepared by freeze-drying. D-Allulose 3-epimerase enzyme preparations comply with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006).

D-Allulose 3-epimerase is not known to be allergenic when used in food processing. Examination of the potential for this enzyme to be a food allergen was made by comparing its amino acid sequence to sequences of known allergens contained within the AllergenOnline allergen database using internationally accepted search criteria. No meaningful identity with known allergens were observed.

Based on the results obtained, oral intake of D-allulose 3-epimerase is not anticipated to pose any allergenicity concern.

2. Description

Yellow to brown liquid or grayish tan powder.

3. Method of manufacture

3.1 Escherischia coli

The genus *Escherichia* was first described by Castellani and Chalmers in 1919 (https://lpsn.dsmz.de/genus/escherichia). *E. coli* is commonly found in the lower intestine of warm-blooded organisms (endotherms). Beside these habitats, certain strains have the potential to cause a wide spectrum of intestinal and extra-intestinal diseases such as urinary tract infection, septicemia, meningitis, and pneumonia in humans and animals. The descendant *E. coli* K-12 is used routinely in molecular biology as both a tool and a model organism. The W3110 sub-strain has long been used as a research organism (Bachmann, 1972). This strain played a critical role in the understanding of the K-12 wild-type strain (Jensen, 1993). Because of the extensive use of the K-12 W3110 strain in research, it has been well characterized, and a highly accurate genomic sequence has been obtained (Hayashi et al., 2006).

The taxonomy of *E. coli* K-12 W3110 is as follows:

Kingdom:	Bacteria
Division:	Proteobacteria
Class:	Gamaproteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Escherichia*
Species:	*E. coli*

Strains of *E. coli* have been deposited in several public culture collections, including the American Type Culture Collection (ATCC, website) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, website). The non-pathogenic strains of *E. coli* are not included on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agent at work or on the list of pathogens in Belgium (EC, 2000; Belgian Biosafety Server, 2008).

Certain strains of *E. coli* can cause intestinal and extra-intestinal diseases in humans and animals. Therefore, *E. coli* was not considered eligible for Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (EFSA, 2014). On the other hand, the K-12 strain of *E. coli* has been safely used in the production of chymosin enzyme, otherwise known as rennin, used in cheese production, for many years without known side effects. The production of rennin by K-12 was affirmed as GRAS by the FDA in 1990. *E. coli* K12 was discussed in a paper published by the FDA in 2006 (Olempska-Beer et al., 2006), in which it is stated "*E. coli* K-12 has been used as a laboratory organism for over 30 years without reported incidents of infection and that it does not produce toxins that cause illness by ingestion, such as Shiga-like toxin produced by certain toxigenic strains of *E. coli*. *E. coli* K-12 has a history of safe use in the production of specialty chemicals and human drugs and was exempted from EPA review under TSCA."

D-Allulose 3-epimerase from *A. globiformis* M30 expressed in *E. coli* K-12 W3110 was affirmed as GRAS by the FDA in 2018 (GRAS No. 624). The Food Safety Commission of Japan (FSCJ) evaluated D-allulose 3-epimerase from *A. globiformis* M30 expressed in genetically modified *E. coli* K-12 W3110 (pWKLP) in 2019 and saw no risk of impairing human health.

3.2 Escherichia coli production strain

The *E. coli* K-12 W3110 production strain, pWKLP, was prepared by transforming the *E. coli* recipient strain with an expression plasmid carrying the structural D-allulose 3-epimerase gene from *A. globiformis* M30 donor, a D-allulose 3-epimerase gene transcription promoter, a repressor and its regulatory region important for the function of the promoter, a terminator, and an antibiotic selection marker. Transformation was performed by the calcium chloride method, followed by selection of the final production. The final production strain was tested for absence of antibiotics. Additionally, the transformation of *E. coli* K-12 W3110 were confirmed by DNA sequencing, as was the stability of the expression plasmid, and the absence of any transformable rDNA. Checks of the reading frames for toxic proteins (Mvir Database Virulence Blast Interface) and allergens (GENTYX gene information software) were negative. The degradability of beta-lactamase was examined by simulation with the ExPAsy PeptideCutter for pepsin, trypsin and chymotrypsin. The acquired data suggests that beta-lactamase is broken down as far as to oligopeptide structures. The presence of antimicrobial resistance gene in the finished product was tested with PCR, targeting beta-lactamase gene fragments specific to the production strain. No such products were detected; therefore, it can be reasonably assumed that no recombinant DNA is present in the final product. The transformation of the intended genes was tested by DNA sequencing of the expression plasmid. The stability of the expression plasmid and the absence of any transformable rDNA were also confirmed.

3.3 Arthrobacter globiformis M30 donor strain

Arthrobacter globiformis is an actinobacterium that is present in soil. It was described by Conn and Dimmick in 1947. They are characterized by pleomorphism (variable shape) and Gram variability (staining positive or negative) although genetically they branch from the Gram-positive phylum Actinobacteria. They have a complex life cycle marked by two distinct stages. When the cultures are young, cells are slender rods that may stain Gram-negative. Jointed rods can be observed after about 1-2 days. By about 30 hours the cells have become very short, gram-positive rods and coccoids. Arthrobacteria are nonsporulating and are members of the actinomycete branch of the gram-positive bacteria.

The wild type *A. globiformis* strain was isolated from soil and strain improvement, using classical colony isolation and selection techniques. The gene encoding the D-allulose epimerase was isolated from *A. globiformis* strain M30, which was deposited in National Institute of Technology and Evaluation (NITE) in Japan under accession number P-1111 (A. Yoshihara et al., 2017). The *A. globiformis* M30 genome was sequenced and the D-AE gene was identified by the amino acid sequence. The gene was cloned from the donor strain and used to construct the expression vector pWKLP.

Several members of the Arthrobacter family, including *A. globiformis*, are present in the microflora of common produce items such as broccoli (Pagada et al., 2000). D-Allulose 3-epimerase from *A. globiformis* M30 expressed in *E. coli* K-12 W3110 was affirmed as GRAS by the FDA in 2018 (GRAS No. 624). *A. globiformis* was listed on the European Food and Feed Cultures Association's (EFFCA) inventory of microorganisms with a documented history of use in human food (IDF, 2002).

3.4 Fermentation, recovery, and formulation

The seed material for the inoculum of the medium for the production of D-allulose 3-epimerase by fermentation is obtained from cultures of the *E. coli* production strain K-12 W3110 (pWKLP) that meet identity, viability, and microbial purity and productivity of the Working Cell Bank (WCB) under controlled conditions. The manufacture of the D-allulose 3-epimerase preparation consists of three steps: pre-culture fermentation, seed fermentation, and main fermentation. Control processes are in place during the fermentation and down-stream processing to prevent a potential microbial contamination and to test for enzyme activity during production.

Fermentation is terminated after optimal enzyme production; D-allulose 3-epimerase is removed from the cells using lysozyme and heat, and then separated from the culture medium containing the

production organism and fermentation media ingredients via a series of filtration steps. The liquid enzyme is concentrated and purified, then formulated into the commercial D-allulose 3-epimerase preparation by the addition of water and sugar alcohols. The powdered product is formulated using a final freeze-drying step. The entire production of D-allulose 3-epimerase preparations is carried out in accordance with GMP and the principles of Hazard Analysis and Critical Control Points (HACCP) using raw materials that are appropriate for food use. The final enzyme preparations contain no major food allergens from the fermentation medium. D-Allulose 3-epimerase preparations are free from mycotoxins and antibiotic activity. D-Allulose 3-epimerase conforms to the General Specifications for Enzyme Preparations used in Food Processing (JECFA, 2006), and the enzyme preparations are tested to be free from the production organism.

4. Identity and characterization

4.1 D-Allulose 3-epimerase

D-Allulose 3-epimerase belongs to the subcategory of epimerases that acts on carbohydrates and derivatives (BRENDA Comprehensive Enzyme Information System). The enzyme is highly specific for D-allulose and epimerizes position C3. It shows very low activity for epimerization of D and L forms of other keto-hexoses, keto-pentoses, and keto-tetroses. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name:	D-allulose 3-epimerase
Other names(s):	psicose epimerase, allulose epimerase, D-psicose 3-epimerase
Reaction:	epimerization of D-fructose at the C3 position to D-allulose and vice-versa
Systematic name:	D-allulose 3-epimerase
EC No.:	5.1.3.30

D-Allulose 3-epimerase produced by *E. coli*, shows very low activity for epimerization of D and L forms of other keto-hexoses, keto-pentoses, and keto-tetroses and no other enzymatic activities. The primary sequence of D-allulose 3-epimerase has been determined to consist of 289 amino acids; its molecular weight, based on SDS-PAGE, is ~32 kDa, with no post-translational modification applied.

Epimerase activity is determined by measuring the production of fructose resulting from the epimerization of allulose, used as a substrate. After a 10-minute reaction, an HPLC technique enables determination of the epimerase activity based on the amount of fructose produced from the substrate.

One unit of epimerase activity is defined as the quantity of enzyme required to produce 1 μmol D-fructose per minute at the specified conditions.

4.2 D-Allulose 3-epimerase enzyme preparation

The D-allulose 3-epimerase preparation is marketed as a liquid formulation or dried product under the trade name Matsurase FE. A representative composition of a commercial liquid formulation of the D-allulose 3-epimerase preparation is provided below:

Enzyme activity:	330 – 450 U/g
Water:	49.4 – 49.9%
Ash:	0.08 – 0.14%
Proteins:	0.36 – 0.48%
TOS*:	4.7 – 4.8%

*includes sugar alcohols

A representative composition of a commercial dried product formulation of the D-allulose 3-epimerase preparation is provided below:

Enzyme activity:	23,000 – 39,000 U/g
Water:	3.8 – 4.2%
Ash:	3.0 – 6.9%
Proteins:	36.2 – 45.5%
TOS:	88.9 – 93.2 %

The D-allulose 3-epimerase preparation consists of the enzyme and substances from the fermentation process; these constitute proteins, peptides, amino acids, carbohydrates, lipids and salt. The components of fermentation are referred to as Total Organic Solids (TOS).

The TOS content of an enzyme preparation is calculated according to the following equation (NAS/NRC, 1981; JECFA, 2006):

$$TOS\ (\%) = 100 - (A + W + D)$$

where

A is the % ash,
Wis the % water and
D is the % diluents and/or other formulation ingredients.

The specifications for D-allulose 3-epimerase include activity (> 330 U/g), lead (≤ 5 mg/kg), total coliforms (≤ 30 CFU/g), *Salmonella* sp. (absent in 25 g), *E. coli* (absent in 25 g), antimicrobial activity (absent by test). D-Allulose 3-epimerase preparation complies with the General Specifications for Enzyme Preparations used in Food Processing as established by the 67th meeting of the Joint Expert Committee on Food Additives (JECFA, 2006).

5. Functional uses

D-Allulose 3-epimerase is intended for use as a processing aid to manufacture D-allulose from D-fructose. The concentration, pH, temperature conditions, and incubation time are optimized to achieve the desired D-allulose concentration. The amount of D-allulose 3-epimerase required to prepare 1 kg of D-allulose is 0.035 kg.

D-Allulose produced by D-allulose 3-epimerase is intended to be used in a wide array of foods, such as cereals, chewing gum, confections and frostings, dressings for salads, jams and jellies, sugars, sugar substitutes (carrier), and various low-calorie or dietetic foods including low-calorie, reduced-calorie, sugar-free beverages (non-alcoholic), cereals, frozen dairy desserts (ice-cream, soft serve, sorbet), yogurt and frozen yogurt, gelatines, pudding and fillings, hard candies, soft candies, and sweet sauces and syrups.

6. Reaction and fate in food

D-Allulose 3-epimerase catalyses the epimerization of D-fructose at the C3 position to D-allulose and vice-versa (Figure 1).

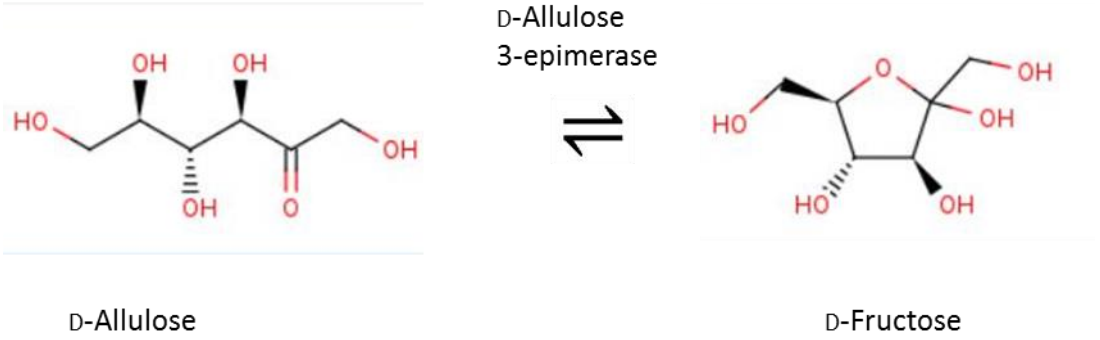

Figure 1. Reaction catalysed by D-allulose epimerase

The enzyme is not expected to have a technological function on the final food and will be denatured or removed. Carry-over of active D-allulose 3-epimerase to the final food product is expected to be negligible. Epimerization activity of D-allulose 3-epimerase results in the formation of D-allulose which is a rare sugar with a similar taste and physical properties to sugar but an energy value that is practically zero.

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