21 June 2021

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for Transglutaminase from *Streptomyces Mobaraensis* M2020197

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Taixing Dongsheng Bio-Tech Co., Ltd., as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that transglutaminase from *Streptomyces mobaraensis* M2020197, is GRAS on the basis of scientific procedures, for use in conventional food and beverage products across multiple categories; these food uses of transglutaminase from *S. mobaraensis* M2020197, are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Taixing Dongsheng Bio-Tech's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the agency.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

Marco Marcucci
R&D Director
Taixing Dongsheng Bio-Tech Co., Ltd.

Email: marco.marcucci@cndsfood.com
Tel: 0086-523-82966333

GRAS NOTICE FOR TRANSGLUTAMINASE FROM *STREPTOMYCES MOBARAENSIS* M2020197

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Taixing Dongsheng Bio-Tech Co., Ltd.
No.1 Tonglian Rd., Huangqiao Town
Taixing City, Jiangsu Province
People's Republic of China
225411

DATE:
21 June 2021

GRAS Notice for Transglutaminase from *Streptomyces mobaraensis* M2020197

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GRAS Notice for Transglutaminase from *Streptomyces mobaraensis* M2020197

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Taixing Dongsheng Bio-Tech Co., Ltd. (TDS Biotech) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that transglutaminase from *Streptomyces mobaraensis* M2020197, as manufactured by TDS Biotech, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on TDS Biotech's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of TDS Biotech, the undersigned hereby certifies that all data and information presented in this notice represents a complete, representative, and balanced submission, and considered all unfavorable as well as favorable information known to TDS Biotech and pertinent to the evaluation of the safety and GRAS status of transglutaminase from *S. mobaraensis* M2020197 as a processing aid in food production, as described herein.

Signed,

21 June 2021

Marco Marcucci
R&D Director
Taixing Dongsheng Bio-Tech Co., Ltd.

Date

1.1 Name and Address of Notifier

Taixing Dongsheng Bio-Tech Co., Ltd.
No.1 Tonglian Rd., Huangqiao Town
Taixing City, Jiangsu Province
People's Republic of China
225411

1.2 Common Name of Notified Substance

The common name of the notified substance is transglutaminase (also referred to as "the food enzyme" throughout this document). Transglutaminase is produced by submerged fermentation of a selected culture of *Streptomyces mobaraensis*. The production strain, *Streptomyces mobaraensis* M2020197, is non-genetically modified and selected for transglutaminase production based on its ability to produce high levels of transglutaminase. The food enzyme is manufactured as an ultra-filtered enzyme concentrate that is standardized with food-grade diluents and carriers, such as maltodextrin, lactose, or dextrose.

1.3 Conditions of Use

Transglutaminase from *Streptomyces mobaraensis* M2020197 is intended for use as a processing aid in the production of meat products, fish products, dairy products, vegetable protein and soybean products, baked goods (including pastries) and bread products, pasta and noodles, grain mixtures, and ready-to-eat cereals in order to improve texture and promote protein binding. A summary of the proposed food categories and use levels for the food enzyme is provided in Table 1.3-1 below. Food uses are organized according to 21 CFR §170.3 (U.S. FDA, 2019a). The maximum recommended use level of the enzyme preparation containing transglutaminase during food processing ranges from 0.05 to 1.5% depending on the food use, while the maximum transglutaminase activity of the enzyme preparation used in food processing applications ranges from 50 to 120 U/g. The maximum use level of transglutaminase on a TOS basis for uses in meat products is 65 mg TOS/kg meat, equivalent to 65 ppm, which is in compliance with the USDA's permitted inclusion level transglutaminase for use as a binding agent in certain standardized and unstandardized meat products (USDA, 2017).

Table 1.3-1 Summary of Food-Uses and Use-Levels of Transglutaminase

Food Use Category	Representative Food Uses[a]	Maximum Recommended Use Level of the Transglutaminase Enzyme Preparation (%)	Maximum Transglutaminase Activity of the Enzyme Preparation (U/g)	Maximum Use Level of Transglutaminase (mg TOS/kg food)[b,c]
Processed meat products	Fresh/cooked/smoked/dry-fermented sausages, cooked hams, meat balls, burgers, liver pate	0.3	120	26.02
Restructured meat and fish products	Beef steak, pork steak, bacon, chicken medallions, turkey medallions, salmon	1.0	90	65.04
Fish-based products	Fish burgers, scallops, shrimps, surimi	0.5	120	43.36
Dairy products	Yoghurt, natural cheese	0.05	120	4.34
	Processed cheese	1.5	90	97.56
	Cream cheese	1.0	90	65.04
	Frozen dairy dessert	0.2	120	17.34
Vegetable protein products	Tofu and soybean products	0.25	110	19.87
	Vegetable burgers, emulsified vegetable sausages	0.2	120	17.34
	Meat substitutes	0.25	120	21.69
Baked goods (including pastries) and bread products	Bread, pizza, croissant, brioche, rolls, cakes, pies, doughnuts,	0.2	120	17.34
Grain mixtures	Burritos, tacos, tortillas	0.2	120	17.34
Pastas and noodles	Pasta and noodles	0.2	120	17.34
Ready-to-eat cereals	Ready-to-eat cereals	0.5	120	43.35

TOS = total organic solids.

[a] Transglutaminase is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[b] Assuming a mean transglutaminase activity of 13.837 U/mg TOS (see Table 2.6.1-1). Calculation: (g enzyme preparation/100 g food) * (U/g enzyme preparation) / 13.837 U/mg TOS * (1,000 g/1 kg).

[c] Assumes 100% of the transglutaminase from *S. mobaraensis* used as a processing aid is carried over into final foods. In reality, foods processed with the enzyme are heat-treated during the food processing (e.g., pasteurization or baking) or cooking by the final consumer, resulting in the inactivation of the enzyme (*i.e.*, the enzyme will have no technological effect in finals foods as consumed). In food products not subjected to heat-treatment during food processing and/or cooking by the consumer prior to consumption, transglutaminase will be inactivated by other factors, such as oxidation, acid or alkaline pH conditions, binding to food matrix *via* cross-linking action, depletion of the available substrate, or inherent factors within the food matrix [*e.g.,* naturally occurring proteolytic enzymes in meats, vegetables, *etc.*].

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2018b), TDS Biotech has concluded that the intended uses of transglutaminase as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Taixing Dongsheng Bio-Tech Co., Ltd.
No.1 Tonglian Rd., Huangqiao Town
Taixing City, Jiangsu Province
People's Republic of China
225411

Should the FDA have any questions or additional information requests regarding this Notification, TDS Biotech will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is TDS Biotech's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the Freedom of Information Act, 5 U.S.C. 552.

Part 2. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity of the Enzyme

Information on the identity of the transglutaminase enzyme is as follows. The transglutaminase is a single polypeptide chain with 331 amino acid residues and a molecular weight of 37.8 kDa. The enzyme is not protein-engineered and is not modified by post-translational processes or by technological procedures. The amino acid sequence was demonstrated to share high structural similarity (>99.7% identity, 100% query coverage) with transglutaminase from *Streptomyces mobaraensis* based on a BLAST search of the non-redundant protein database of NCBI.

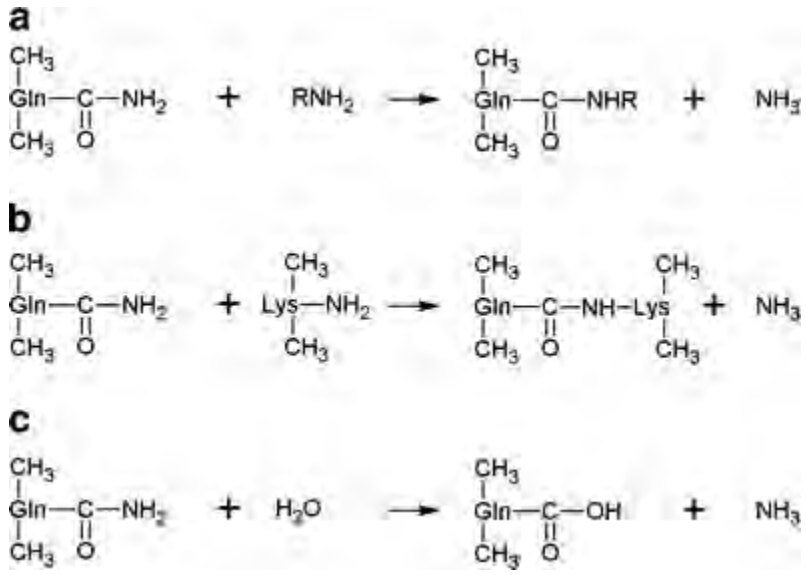
Accepted Name:	Transglutaminase
Systematic Name:	protein-glutamine:amine γ-glutamyltransferase
Synonyms:	Factor XIIIa; fibrinoligase; fibrin stabilizing factor; glutaminylpeptide γ-glutamyltransferase; polyamine transglutaminase; tissue transglutaminase; R-glutaminyl-peptide:amine γ-glutamyl transferase
Enzyme Commission (EC) Number:	EC 2.3.2.13
CAS Number:	80146-85-6
Amino Acid Sequence:	DSDERVTPPAEPLDRMPDPYRPSYGRAETIVNNYIRKWQQVYSHRD GRKQQMTEEQREWLSYGCVGVTWVNSGQYPTNRLAFAFFDEDKYKN ELKNGRPRSGETRAEFEGRVAKDSFDEAKGFQRARDVASVMNKALE NAHDEGAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKDRN GGNHDPSKMKAVIYSKHFWSGQDRSGSSDKRKYGDPEAFRPDRGTG LVDMSRDRNIPRSPTSPGESFVNFDYGWFGAQTEADADKTVWTHGN HYHAPNGSLGAMHVYESKFRNWSDGYSDFDRGAYVVTFVPKSWNTA PDKVTQGWP

2.2 Enzyme Activity

2.2.1 Principal Enzymatic Activity

The transglutaminase enzyme has two types of substrates: polypeptides or proteins with glutamine or lysine. The major substrates of transglutaminase are polypeptides or proteins with lysine residues. Transglutaminase catalyzes the acyl-transferase reaction using peptide-bonded glutamine residues as acyl donors and primary amines as acceptors (reaction 1; Figure 2.2.1-1a) (Lorand and Graham, 2003; de Souza *et al.,* 2011; Kieliszek and Misiewicz, 2014). If the epsilon-amino group of lysine acts as the primary amine, epsilon-(gamma-Glu) Lys bonds are formed, resulting in an intra- and inter-molecular cross-linking of proteins (reaction 2; Figure 2.2.1-1b). In the absence of amino substrates, transglutaminase catalyzes the deamidation of glutamyl residues in which water is used as acyl acceptors (reaction 3; Figure 2.2.1-1c). This side reaction is minor compared to the primary enzymatic reaction.

Figure 2.2.1-1 Enzymatic Reactions of Transglutaminase: (a) Acyl-Transfer Reaction, (b) Cross-Linking Reaction, and (c) Deamidation Reaction

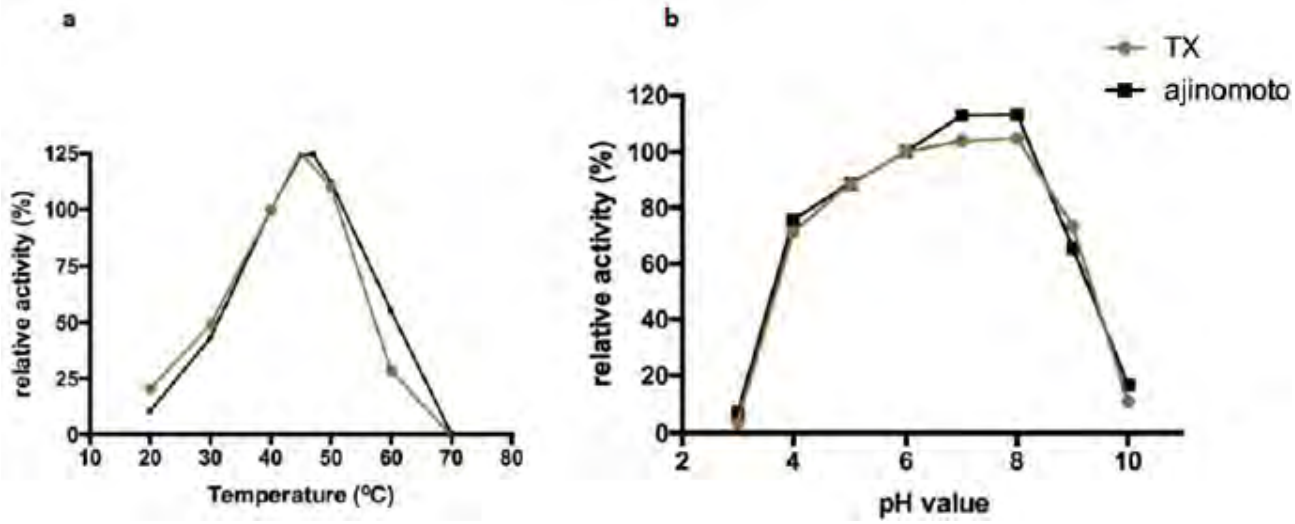


Source: Kieliszek and Misiewicz (2014).

2.2.2 Optimal Reaction Conditions

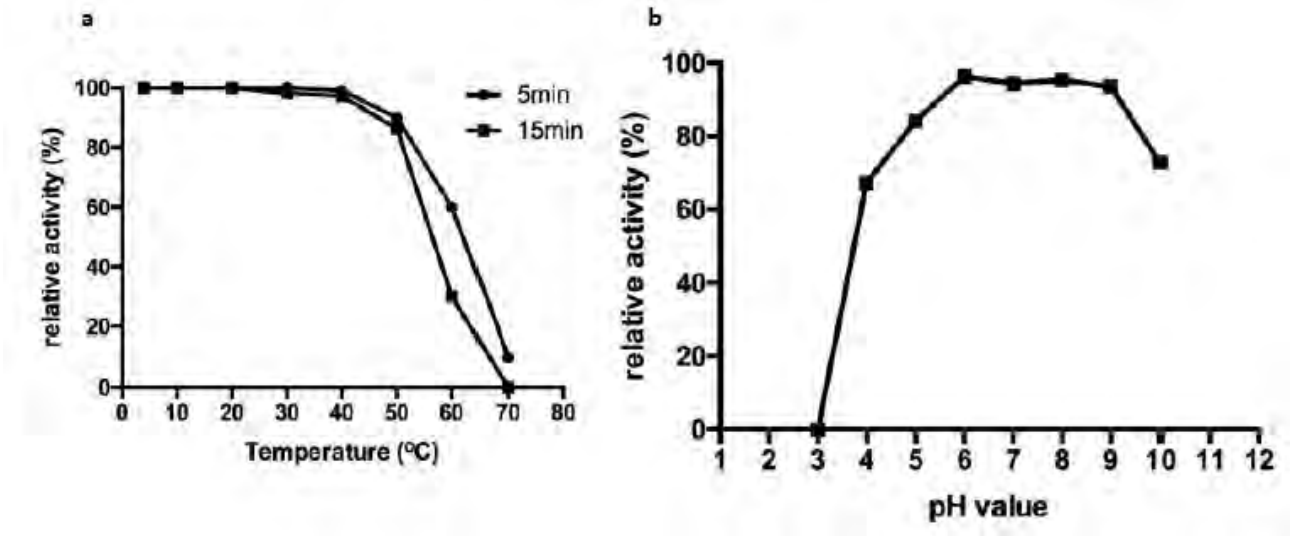
Transglutaminase from *S. mobaraensis* M2020197 has optimal enzymatic activity at pH 5.0 to 8.0 and temperatures of 45 to 55°C (Figure 2.2.2-1). The optimal temperature and pH of the transglutaminase from *S. mobaraensis* M2020197 are similar to a commercially available transglutaminase preparation derived from *Streptoverticillium mobaraense*, which has been the subject of several GRAS notifications (*e.g.*, GRNs 4, 29, 55, and 95) (U.S. FDA, 1998, 1999, 2001, 2002).

Figure 2.2.2-1 Optimal (a) Temperature and (b) pH Conditions for Transglutaminase Derived from *Streptomyces mobaraensis* Strain M2020197



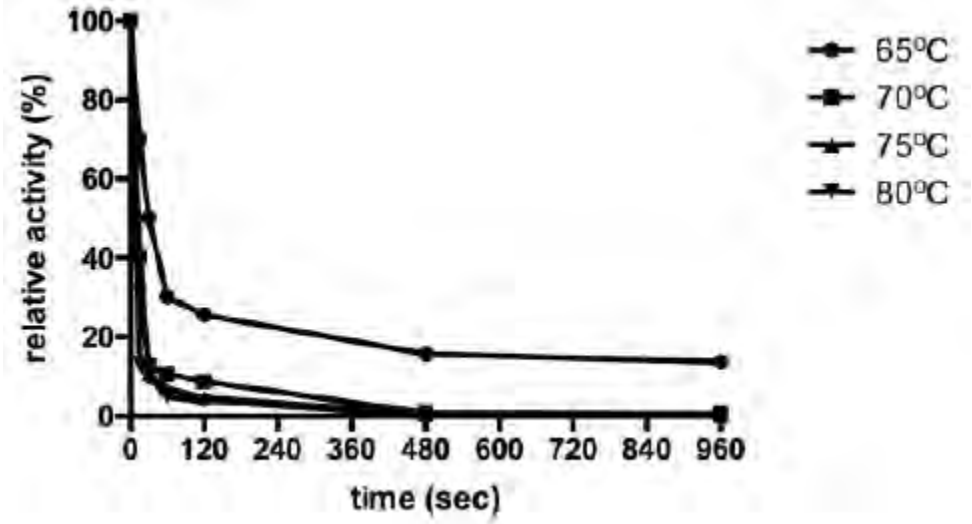
The temperature and pH stability of the transglutaminase from *S. mobaraensis* M2020197 was determined analytically using the internal method of analysis for transglutaminase activity described above in Section (2.1.3.1). The transglutaminase food enzyme was stable at temperatures up to approximately 50°C and between pH 5.0 to 9.0 (Figure 2.2.2-2). The enzyme activity gradually declined above 60°C and had no activity at 70°C, suggesting that the enzyme may be inactivated at temperatures greater than 70°C when held for 5 minutes.

Figure 2.2.2-2 Transglutaminase Activity Under Various (a) Temperature Conditions and (b) pH Conditions



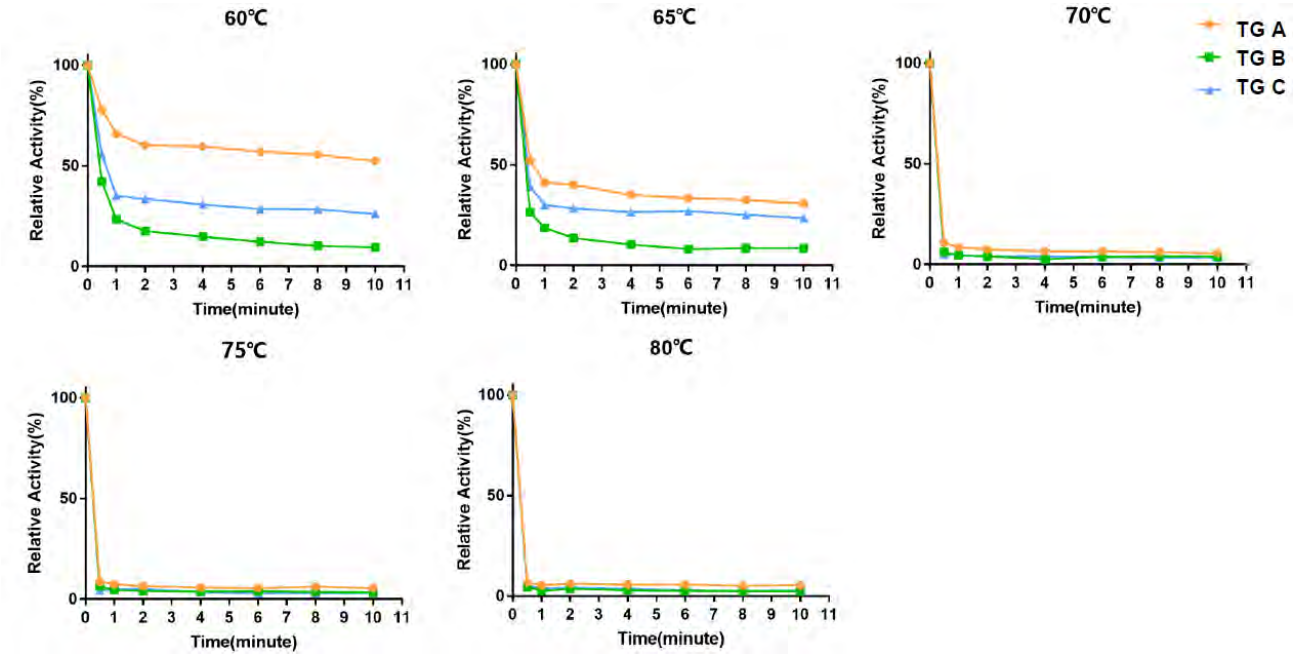
In addition, the enzyme activity of transglutaminase from *S. mobaraensis* M2020197 was investigated at temperatures of 65, 70, 75, and 80°C when held for up to 16 minutes (Figure 2.2.2-3). The transglutaminase activity was negligible after approximately 1 minute at temperatures over 70°C, while enzyme activity was approximately 30% of the initial activity at 65°C after 1 minute.

Figure 2.2.2-3 Transglutaminase Activity Following Heating at Temperatures 65 to 80°C for Up to 16 Minutes



The enzyme activity of 3 batches of transglutaminase from *S. mobaraensis* M2020197 was also investigated at 60, 65, 70, 75, and 80°C for up to 10 minutes. The results are presented in Figure 2.2.2-4 and are consistent with the findings described above. Transglutaminase activity gradually drops to ~50% after 1 minute at 60 and 65°C, and is completely deactivated after 0.5 minutes at temperatures greater than 70°C.

Figure 2.2.2-4 Transglutaminase Activity Following Heating at Various Temperatures for up to 10 Minutes



2.3 Description of the Production Organism

The transglutaminase food enzyme is derived from a non-genetically modified strain of *Streptomyces mobaraensis* (strain M2020197). The identity of the production strain was confirmed using 16S rDNA sequencing to be *S. mobaraensis*. *S. mobaraensis*, also referred to as *Streptoverticillium mobaraense*[1], has been used as a source organism for transglutaminase for decades. Microbial transglutaminase from *S. mobaraense* was concluded to be GRAS for its intended use in a variety of food products, including processed cheeses, yogurt, frozen desserts, vegetable protein products and meat substitutes, processed seafood, pasta, pastries, ready-to-eat cereal products, and grain mixtures. The GRAS conclusions were filed without objection by the FDA under GRNs 4, 29, 55, and 95 (U.S. FDA, 1998, 1999, 2001, 2002). *S. mobaraensis* M2020197 has been characterized and demonstrated to be non-pathogenic and non-toxigenic and is considered to be a suitable source organism for transglutaminase (see Section 6.2 for further details).

2.4 Manufacturing Process

2.4.1 Raw Materials and Processing Aids

All raw materials and processing aids comply with food-grade specifications as established in the Food Chemicals Codex (FCC) or equivalent international food or pharmacopeia standard (*e.g.,* United States

[1] https://www.uniprot.org/taxonomy/35621.

Pharmacopeia), and are permitted for use in food by U.S. federal regulations or are GRAS for their respective uses. All filtration aids are those commonly used by the food industry in the purification of food ingredients.

2.4.2 Overview of the Manufacturing Process

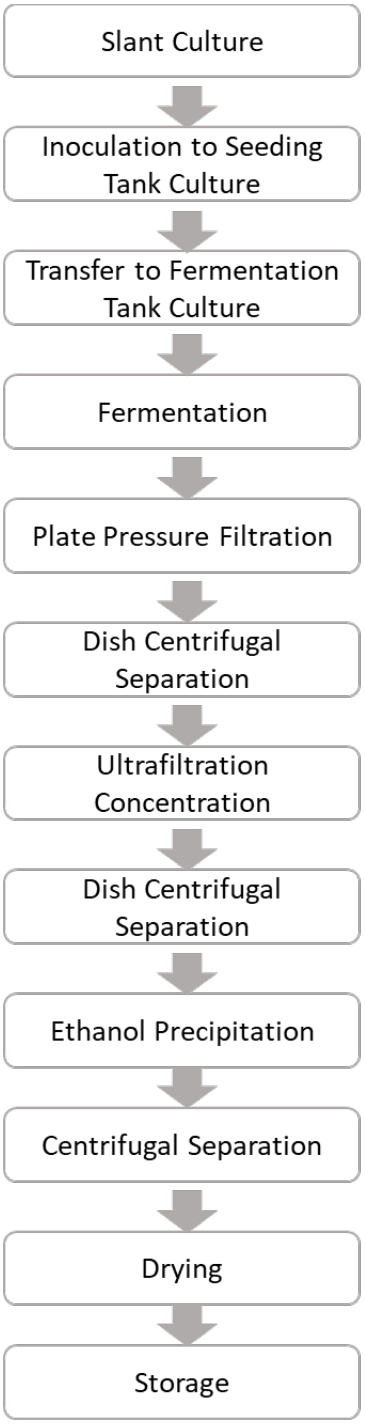
TDS Biotech manufactures transglutaminase by submerged fermentation of a selected culture of *Streptomyces mobaraensis* M2020197. The food enzyme is produced using food-grade materials and using quality-controlled fermentation and purification/recovery processes that are common to the food enzyme industry. The food enzyme is manufactured in accordance with cGMP, HACCP, and FSSC 22000/ISO 22000. A schematic overview of the manufacturing process for the transglutaminase food enzyme is presented in Figure 2.4.2-1.

TDS Biotech maintains a master culture of a wildtype strain of *S. mobaraensis* at -30°C. The fermentation process consists of 3 steps: slant culture, seed culture, main fermentation. During the slant culture and seed tank culture steps, the wildtype strain culture is inspected to ensure the absence of other microbial contaminants. The slant culture is subject to shake flask fermentation, and the enzyme activity and growth conditions are characterized. Cultures showing high transglutaminase activity are isolated and selected as the production strain culture. This process is repeated on at least 6 batches of wildtype strain to ensure the stability of the production strain. The production strain culture is inspected for microbial impurities, enzyme activity, yield, and specific enzyme activity prior to use in the main fermentation.

Transglutaminase is produced by the production organism during the main fermentation. The fermentation conditions are kept for optimal growth of the production organism and biosynthesis of transglutaminase. Following the fermentation period, the fermentation broth is subject to plate pressure filtration (pressure >0.1 MPa), dish centrifugal separation, and then concentrated by ultrafiltration. After the ultrafiltration step, the solution is subjected to a second dish centrifugal separation step and the enzyme is precipitated by the addition of ethanol[2] and centrifuged. The resulting solution containing the transglutaminase food enzyme is freeze dried and stored.

[2] Ethanol temporarily denatures the enzyme, thus precipitating the enzyme. However, once ethanol is removed by centrifugation and drying/evaporation, the enzyme is rehydrated and recovers its original folding structure, and therefore, resuming its catalytic activity.

Figure 2.4.2-1 Manufacturing Process of Transglutaminase from *Streptomyces mobaraensis* M2020197



2.4.3 Process Controls and Quality Control Measures

A HACCP plan is in place for the manufacture of the transglutaminase food enzyme produced from *S. mobaraensis* M2020197 in which the critical control points have been identified and measures set in place for the prevention of the identified hazards. Quality control steps for these critical control points have been included as part of this plan in order to ensure adherence with the established manufacturing process and to produce a high quality and consistent product. These include steps to reduce microbial contaminants in

the fermentation medium and foreign body detection of the final product. All components of the fermentation media are sterilized prior to use. The final product is passed through an X-ray detector to ensure the absence of foreign matter such as metal, glass, and ceramics, in the final product. Each manufactured batch of the food enzyme is analyzed for conformity to the specifications for the food enzyme. Batches that do not meet the specifications for the food enzyme are not released for further processing or for the formulation of final enzyme preparations.

2.5 Product Specifications

Specifications for the transglutaminase food enzyme produced from *S. mobaraensis* M2020197 have been established that comply with the purity and microbial limits established for enzyme preparations by JECFA (2006) and FCC (2018) (Table 2.5-1). All methods of analysis are internationally recognized or validated methods.

Table 2.5-1 Specifications for the Transglutaminase Food Enzyme from *Streptomyces mobaraensis* Strain M2020197

Specification Parameter	Specification	Method of Analysis
Lead	NMT 5 mg/kg	GB 5009.12-2010 (Method I)
Arsenic	NMT 3 mg/kg	GB/T 5009.11-2003 (Method I)
Escherichia coli	Negative in 25 g	GB 4789.38-2012 (Method II)
Total Coliforms	NMT 30 CFU/g	GB 4789.3-2010 (Method II)
Salmonella sp.	Negative in 25 g	GB 4789.4-2010
Antimicrobial Activity	Negative	JECFA Monograph 6

CFU = colony-forming units; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NMT = not more than.

2.6 Product Analyses of the Enzyme

2.6.1 Batch Analyses

Analytical data on 6 production batches of transglutaminase concentrate from *S. mobaraensis* M2020197 (Lot Nos. H20141247, H20141252, H20151114, 20200287, 20200289, 20200290) demonstrate that the manufacturing process produces a consistent product that meets the product specifications (Table 2.6.1-1). Heavy metal contaminants (*i.e.*, arsenic and lead) were below the specification limits of 3 mg/kg and 5 mg/kg, respectively. In addition, microbiological contaminants were also below the limit of detection and/or in compliance with the specifications for the food enzyme. All batches were negative for antimicrobial activity.

Table 2.6.1-1 Summary of the Product Analysis for 6 Lots of Transglutaminase from *Streptomyces mobaraensis* Strain M2020197

Specification Parameter	Specification Limit	Manufacturing Lot No.					
		H20141247	H20141252	H20151114	20200287	20200289	20200290
Lead (mg/kg)	NMT 5 mg/kg	0.012	0.011	0.010	0.06	0.08	0.07
Arsenic (mg/kg)	NMT 3 mg/kg	0.19	0.21	0.21	0.02	0.03	0.02
Heavy Metals (as Pb) (mg/kg)	NMT 5 mg/kg	<5	<5	<5	NM	NM	NM
Ethanol (mg/kg)	NMT 10 mg/kg	ND[a]	ND[a]	ND[a]	NM	NM	NM
Total Coliforms (CFU/g)	NMT 30 CFU/g	<10	<10	<10	<10	<10	<10
Escherichia coli (CFU/g)	Negative in 25 g	ND	ND	ND	<10	<10	<10
Salmonella (Negative/25 g)	Negative in 25 g	ND	ND	ND	ND	ND	ND
Antimicrobial Activity	Negative	ND	ND	ND	NM	NM	NM

CFU = colony-forming units; ND = not detected; NM = not measured.
[a] Limit of detection = 10 mg/kg.

2.6.2 Additional Characterization of the Enzyme

2.6.2.1 Composition of the Enzyme Preparation

Compositional data on 6 production batches (Lot Nos. H20141247, H20141252, H20151114, 20200287, 20200289, 20200290) of the ultra-filtered enzyme concentrate of transglutaminase from *S. mobaraensis* M2020197 are provided in Table 2.6.2.1-1. The transglutaminase food enzyme is composed of approximately 85% protein, 5.8% ash, and 3.1% water. The mean transglutaminase activity is 12,592 U/g or approximately 14 U/mg TOS.

Table 2.6.2.1-1 Compositional Analysis for 6 Lots of Transglutaminase from *Streptomyces mobaraensis* Strain M2020197 (Dried Ultra-Filtered Concentrate)

Specification Parameter	Manufacturing Lot No.					
	H20141247	H20141252[a]	H20151114[a]	20200287	20200289	20200290
Transglutaminase Activity (U/g)	13,851	13,742	10,184	11,287	12,260	11,683
Protein (g/100 g)	85.3	85.0	85.2	NM	NM	NM
Ash (g/100 g)	5.8	5.7	6.0	NM	NM	NM
Moisture (g/100 g)	3.0	3.2	3.1	NM	NM	NM
Total Organic Solids[b] (g/100 g)	91.2	91.1	90.9	NM	NM	NM
Activity (U/mg TOS)	15.186	15.085	11.241	NM	NM	NM

NM = not measured; TOS = total organic solids.
[a] Batch used in the toxicology studies.
[b] Calculated as 100 - (ash + water). The enzyme preparation can contain diluents and carriers, such as maltodextrin, lactose or dextrose.

2.6.2.2 Mycotoxins

Three production batches of transglutaminase concentrate from *S. mobaraensis* M2020197 (Lot Nos. 20200287, 20200290, 20200289) were analyzed for mycotoxins. Based on the results of this analysis, aflatoxins (B1, B2, G1, G2), ochratoxin A, zearalenone, sterigmatocystin, and T2 Toxin were not detected in the enzyme preparation (Table 2.6.2.2-1).

Table 2.6.2.2-1 Mycotoxins and Secondary Metabolites Analyses for 3 Lots of Transglutaminase from *Streptomyces mobaraensis* Strain M2020197 (Dried Ultra-Filtered Concentrate)

Mycotoxin	LOQ	Manufacturing Lot No.			Method of Analysis
		20200287	20200290	20200289	
Aflatoxin B1	0.1 µg/kg	ND	ND	ND	GB 5009.22-2016 (3rd Method)
Aflatoxin B2	0.03 µg/kg	ND	ND	ND	
Aflatoxin G1	0.1 µg/kg	ND	ND	ND	
Aflatoxin G2	0.03 µg/kg	ND	ND	ND	
Ochratoxin A	1 µg/kg	ND	ND	ND	GB 5009.96-2016
Zearalenone	20 µg/kg	ND	ND	ND	GB 5009.209-2016 (1st Method)
Sterigmatocystin	10 µg/kg	ND	ND	ND	Internal Method [CON-PV 01126 (2020-05)]
T2 Toxin	10 µg/kg	ND	ND	ND	NY/T 2071-2011

LOQ = limit of quantification; ND = not detected

2.6.2.3 Minerals

Three production lots (Lot Nos. H20141247, H20141252, H20151114) of transglutaminase concentrate from *S. mobaraensis* M2020197 were analyzed for residual minerals (calcium, magnesium, iron, and sodium) that may be carried over from the fermentation medium. The results are summarized in Table 2.6.2.3-1.

Table 2.6.2.3-1 Summary of the Mineral Analysis for 3 Lots of Transglutaminase from *Streptomyces mobaraensis* Strain M2020197

Mineral	Manufacturing Lot No.		
	H20141247	H20141252	H20151114
Calcium (mg/100 g)	233	229	241
Magnesium (mg/100 g)	103	112	98
Iron (mg/100 g)	88	89	99
Sodium (mg/100 g)	2,278	2,297	2,451

2.6.2.4 Free Amino Acids

The same 3 production lots (Lot Nos. H20141247, H20141252, H20151114) of transglutaminase concentrate from *S. mobaraensis* M2020197 were analyzed for the presence of free amino acids, calculated as the difference between total amino acids and protein content. The results demonstrate the levels of free amino acids to be in the range of 4.93 to 4.94 g/100 g across 3 lots of the food enzyme.

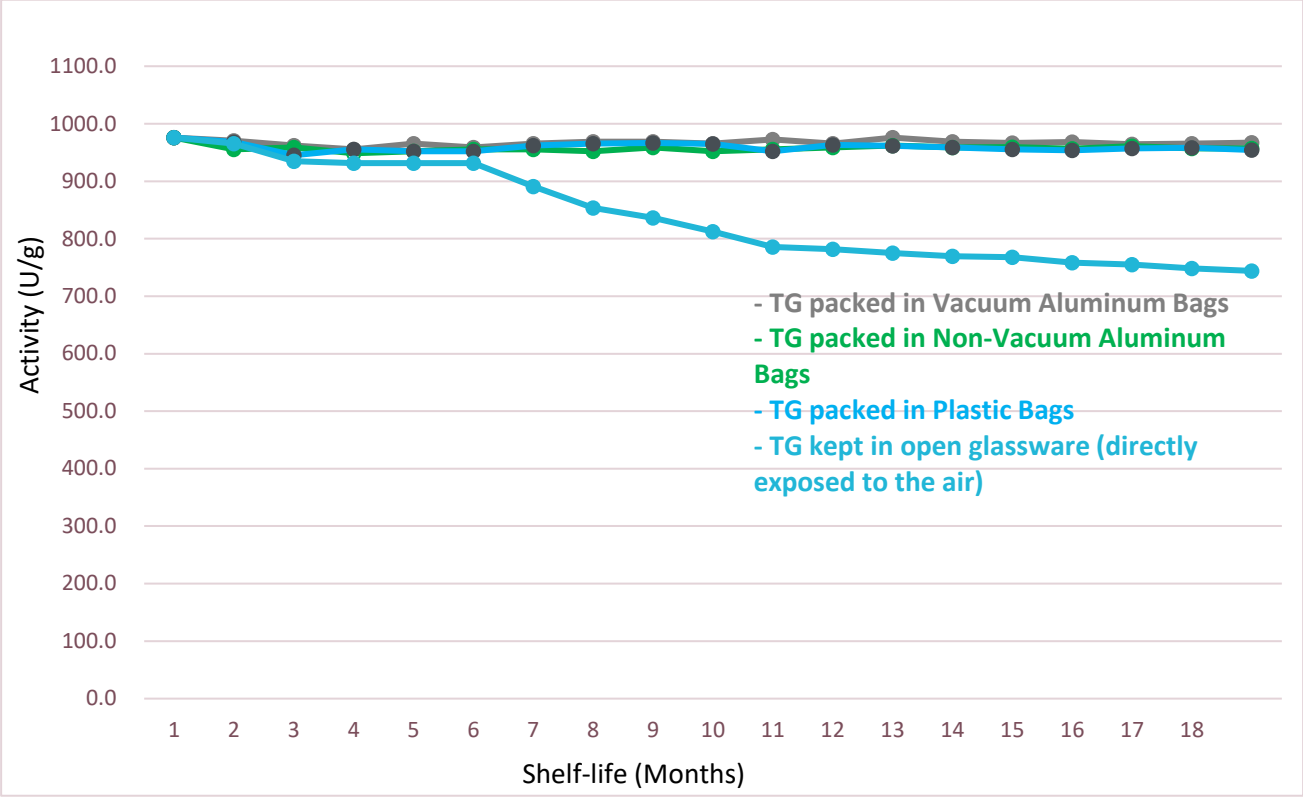
2.6.2.5 Secondary Enzyme Activity

The same 3 production lots (Lot Nos. H20141247, H20141252, H20151114) of transglutaminase concentrate from *S. mobaraensis* M2020197 were analyzed for protease activity. The results demonstrate the absence of protease activity across 3 lots of the food enzyme.

2.7 Storage Stability of the Enzyme

The shelf-life stability of transglutaminase from *S. mobaraensis* M2020197 was investigated following storage for 18 months in an aluminum foil bag with and without vacuum, plastic zip-lock bag, and open glassware. The temperature and relative humidity were maintained at 21±2°C and 58±9%, respectively. The results are summarized in Figure 2.7-1 and demonstrate that the enzyme was stable when stored in aluminum or plastic bags for 18 months, as no appreciable changes in transglutaminase activity was observed. The enzyme was demonstrated to be stable for about 6 months when stored in the open glassware, as shown by decreases in activity after the 6-month timepoint.

Figure 2.7-1 Transglutaminase Activity After Storage Under Shelf-Life Conditions in Aluminum Foil Bags (With and Without Vacuum), Plastic Bag, and Open Glassware



Part 3. § 170.235 Dietary Exposure

3.1 Physical or Technical Effect

Transglutaminase catalyzes acyl transfer reactions between γ-carboxamide groups of protein-bound glutamine and primary amines. If the epsilon-amino group of lysine acts as the primary amine, epsilon-(gamma-Glu)Lys bonds are formed, resulting in an intra- and inter-molecular cross-linking of proteins. In the absence of free amine groups, deamidation occurs as a minor side reaction. The primary enzymatic activity of transglutaminase is the cross-linking of protein. TDS Biotech intends to use transglutaminase in processed meat and fish products, dairy products (including yogurt, cheese, and frozen desserts), baked goods, pastries, bread products, pasta and noodles, grain mixtures, ready-to-eat cereals, and vegetable protein products (including soybean products such as tofu and meat substitutes/alternatives). Transglutaminase is already permitted for use at levels of up to 65 ppm as a binder in certain standardized meat food products in the U.S. (such as fabricated steak, roast beef parboiled and steam roasted, and sausage (USDA/FSIS, 2020), in unstandardized meat products, in meat products formulated to reduce sodium or fat content, and in binding pieces of whole muscle meat to fabricate or reform cuts of meat (USDA/FSIS, 2001).

Transglutaminase-processed food products undergo heat treatment at temperatures greater than 75°C (*e.g.*, cooking, baking, pasteurization) prior to consumption of the final food, resulting in the inactivation of the enzyme. In food products not subjected to heat-treatment during food processing and/or cooking by the consumer prior to consumption, transglutaminase will be inactivated by other factors, such as oxidation, acid or alkaline pH conditions, binding to food matrix *via* cross-linking action, depletion of the available substrate, or inherent factors within the food matrix (*e.g.,* naturally occurring proteolytic enzymes in meats, vegetables, *etc.*). The use of transglutaminase is considered self-limiting since the substrate is protein and use levels which exceed the amount required to obtain the intended technological effect are reported to result in the decline of the organoleptic quality of the food (Ajinomoto USA, 2001).

The catalytic reaction of transglutaminase is ceased by depletion of substrate protein during food processing; the presence of the enzyme at low levels is not expected to pose a safety concern as it does not contain any functionality in the absence of substrate protein. Furthermore, residual amounts of transglutaminase would be readily digested by the gastric fluid following ingestion, such that intact transglutaminase would not be present for absorption into the systemic circulation. A summary of the technological function of transglutaminase in each of the proposed food use is provided in Table 3.1-1.

Table 3.1-1 Technological Function of Transglutaminase Under its Proposed Food Use

Food Use	Technological Function
Meat and Fish-Processed Food Products	Improvement in the texture and consistency of food products. The enzyme is inactivated and heat-denatured at the end of processing by heat treatment (*e.g.,* cooking).
Dairy Products (Yogurt, Cheese, Frozen Dessert)	Reaction with casein present in dairy products to improve the texture and rheological stability of the product. The enzyme is inactivated and heat-denatured by pasteurization or due to the low pH environment caused by *Lactobacillus* fermentation, as well as by the proteolytic activity of proteases secreted by *Lactobacillus* species during the fermentation.
Baked Goods, Pastries, Bread Products, Pasta and Noodles, Grain Mixtures, and Ready-To-Eat Cereal	Reaction on the gluten and other protein structure to improve functionality of the structures. The enzyme is inactivated and heat-denatured in the baking process.
Vegetable Protein Products (including Tofu, Soybean Products, and Meat Substitutes or Alternatives)	Increase strength and deformation resistance of vegetable protein products. The enzyme is inactivated by heat treatment during processing (maximum 90°C).

TDS Biotech has developed an internal analytical method to detect the level of residual transglutaminase activity in the finished food products immediately after production. The finished food products tested with this analytical method, as well as information on their preparation are summarized in Table 3.1-2. The foods selected for analysis were either processed with heat treatment (*e.g*., cooked emulsified sausage) or without heat treatment (*e.g*., reformed raw meat, cheese and yogurt). All foods were treated with transglutaminase from *S. mobaraensis* M2020197 at higher use levels than those proposed in Table 1.3-1 to replicate worst-case exposures. Samples of the finished foods were collected after overnight storage to test for residual enzyme activity. In addition, a negative control with no transglutaminase added, and 3 positive control tubes containing transglutaminase with activity levels of 0.01, 0.001, and 0.0001 U/mL were used as comparators.

Table 3.1-2 Preparation of Finished Food Samples Treated with Transglutaminase

Finished Food	Processing Conditions	Transglutaminase Activity and Use Level	Final Transglutaminase Activity After Food Processing and Dilution[a]
Reformed raw meat	The product was treated with transglutaminase during processing, then packaged into casings and chilled overnight at 2 to 4°C. The final food product was not subject to heat treatment.	Use level of 1% Activity of 90 U/g	0.09 U/g
Cooked emulsified sausage	Sausages were prepared using a standard recipe and transglutaminase. Sausages were stuffed into casings and cooked for 45 minutes at 72°C, cooled, and stored overnight at 2 to 4°C.	Use level of 0.2% Activity of 150 U/g	0.03 U/g
Yogurt	Yogurt was prepared by inoculating *Lactobacillus* cultures in full fat milk, followed by pasteurization, cooling, and treatment with transglutaminase. Fermentation was stopped once a pH of 4.6 was achieved, and the yogurt products were stored overnight at 2 to 4°C.	Use level of 0.025% Activity of 150 U/g	0.0075 U/g

Table 3.1-2 Preparation of Finished Food Samples Treated with Transglutaminase

Finished Food	Processing Conditions	Transglutaminase Activity and Use Level	Final Transglutaminase Activity After Food Processing and Dilution[a]
Cheese	Cheese was prepared by inoculating *Lactobacillus* cultures in skimmed milk, followed by pasteurization, cooling, and treatment with transglutaminase. Fermentation was stopped once a pH of 4.6 was achieved, and the product was heated to 50 to 55°C to improve curd texture and to separate it from the whey. The product was stored overnight at 2 to 4°C.	Use level of 0.03% Activity of 150 U/g	0.009 U/g

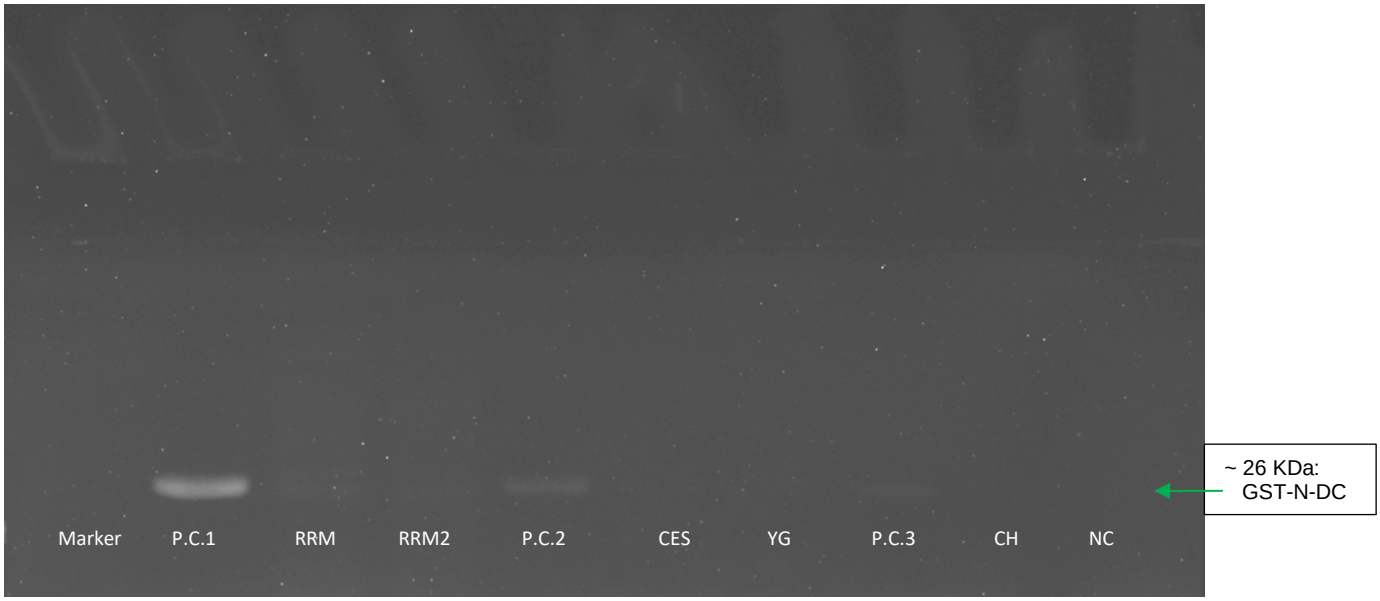
[a] The final enzyme activity after the processing of food samples was calculated by multiplying the use level and activity of the transglutaminase. A dilution factor of 10 was applied for reformed raw meat and sausage samples, and a dilution factor of 5 was applied for yogurt and cheese samples.

To quantify transglutaminase activity in these final food samples, modified glutathione-S-transferase (GST) protein and an autofluorescent dye (dansylcadaverine) were added as substrates for the transglutaminase enzyme to the homogenized samples of the selected foods. The glutamine residues in the native GST protein are not recognizable by transglutaminase, thus, the protein was modified by adding a short amino acid sequence containing a reactive glutamine residue (acyl-donor) to the *N*-terminus. Dansylcadaverine was used as the acyl-acceptor due to the available primary amine.

The food samples and substrates were mixed in reaction tubes with a shaker for 2 hours at 37°C, followed by heating to 95°C for 5 minutes to stop the catalysis reaction. The transglutaminase-mediated catalysis of the 2 substrates (modified GST and dansylcadaverine) results in a GST-dansylcadaverine conjugate protein with a molecular weight of 26 kDa. The conjugate protein was purified from the food samples and separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The residual transglutaminase activity was then quantified by analyzing the gel *via* spectrophotometry due to the fluorescent properties of dansylcadaverine; the resulting conjugate protein exhibits maximum absorption at 335 nm and maximum emission at 525 nm. The results of the gel analyses are discussed below.

The SDS-PAGE gel analyzed by spectrophotometry did not show any bands at approximately 26 kDa, the molecular weight of the conjugate protein, in the samples of cooked sausage, yogurt, or cheese (lanes CES, YG, and CH in Figure 3.1-1 below). The presence of the conjugate protein in the samples would indicate transglutaminase activity, as described in the reaction above. Thus, the absence of bands at 26 kDa in these samples demonstrates a lack of transglutaminase activity or negligible activity below the established limit of detection of 0.0001 U/mL. In contrast, progressively faint bands corresponding with decreasing concentrations of transglutaminase were visible at 26 kDa for each of the 3 positive control groups (lanes P.C.1, P.C.2, and P.C.3). Slight enzymatic activity was observed in the lane containing the reformed raw meat sample (lane RRM), with a band much lower in intensity than the second positive control group (0.001 U/mL). Considering that the original enzyme activity in the reformed raw meat sample was calculated to be 0.09 U/mL after processing and dilution, the resulting band at a lower intensity than the 0.001 U/mL positive control indicates that at least 90% of the original enzymatic activity was reduced. This suggests that transglutaminase was inactivated due to other factors in the absence of heat treatment. For further analysis, the same reformed raw meat production batch was tested again in the same manner as described above after a second day of cold storage, and no bands were observed in the gel (lane RRM2). Thus, the results obtained using TDS Biotech's analytical method demonstrates that the transglutaminase enzyme used at high levels as a processing aid in foods is effectively inactivated during the production process, both with and without heat treatment steps.

Figure 3.1-1 SDS-PAGE Gel Containing Samples of Reformed Raw Meat (RRM, RRM2), Cooked Emulsified Sausage (CES), Yogurt (YG), and Cheese (CH) Analysed with Spectrophotometry



NC: Negative control; PC: Positive control

The same batches of food samples were also analyzed using a commercial kit, the XEMATest MTG, which is a rapid immunochromatographic strip test currently used for the qualitative determination of transglutaminase in food products on the market. The kit can be used to determine the presence of transglutaminase; however, it cannot distinguish between active and inactive forms of the enzyme. As demonstrated by the results of this analysis, the transglutaminase enzyme was detected in positive control 1 (0.01 U/mL), positive control 2 (0.001 U/mL), and both reformed raw meat samples (Figure 3.1-2), indicated by the red band. A single red band indicates a "negative" result. The limit of detection for this kit is also lower than the limit of detection for TDS Biotech's internal method (0.0001 U/mL) since the presence of transglutaminase was not detected by the kit in positive control 3 (activity of 0.0001 U/mL).

Figure 3.1-2 XEMATest MTG Results for the Qualitative Determination of Transglutaminase in Samples of Reformed Raw Meat (RRM1, RRM2), Cooked Emulsified Sausage (CES), Yogurt (YG), and Cheese (CH)

NC: Negative control; PC: Positive control

Thus, the XEMATest MTG kit detected the presence of transglutaminase in the reformed raw meat samples, while TDS Biotech's internal method of analysis was used to determine that the transglutaminase present in these samples was inactive.

In summary, analysis using the internal method developed by TDS Biotech demonstrated transglutaminase to be effectively inactivated following processing under standard food processing conditions, with and without heat treatment steps.

3.2 Estimated Dietary Intake of Transglutaminase

3.2.1 Methodology

The potential human exposure to transglutaminase was estimated using the Budget Method, which is a widely-accepted preliminary screening tool used to assess the intake of chemicals such as food additives (FAO/WHO, 2009). The Budget Method allows for the calculation of a theoretical maximum daily intake (TMDI) based on assumptions regarding the maximum human physiological levels of daily food and beverage consumption, rather than on food consumption data collected from dietary surveys. Specifically, the Budget Method relies on conservative assumptions made regarding (i) the level of consumption of solid foods and of non-milk beverages, (ii) the level of presence of the substance in solid foods and in non-milk beverages, and (iii) the proportion of solid foods and of non-milk beverages that may contain the substance (FAO/WHO, 2009). The levels of anticipated exposure to food enzymes that are derived using the Budget Method are thus considered to be conservative estimates (FAO/WHO, 2009). The results of this assessment are described in Section 3.2.2 below.

3.2.2 Estimated Daily Intake for the General Population

3.2.2.1 Assumptions of the Budget Method

Level of Consumption of Solid Foods

The Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) report on the *Principles and Methods for the Risk Assessment of Chemicals in Food* (FAO/WHO, 2009) specifies the standard values for food intakes to be 0.05 kg/kg body weight/day for solid foods.

Level of Presence of Food Enzyme in Solid Foods

To estimate the exposure to transglutaminase from its intended uses in foods containing ingredients made with the enzyme, it is assumed that the entire enzyme preparation added during processing will be present in the final foods as consumed (*i.e.*, assuming no removal and/or inactivation). Thus, the amount of enzyme assumed to be present in solid foods is based on the maximum level of the enzyme used in the production of final foods. It is assumed that all of the transglutaminase added during food processing remains in the final food as intended to be consumed. Therefore, the maximum amount of transglutaminase that could potentially be present in foods processed with the enzyme is 97.56 mg TOS/kg food.

Proportion of Solid Foods That May Contain the Food Enzyme

According to the FAO/WHO report, the default proportions that are typically assumed are that 12.5% of all solid foods consumed will contain the food enzyme (FAO/WHO, 2009). However, the proportion of solid foods containing the food enzyme may be increased to 25% in cases where the enzyme is used in a wide range of food categories (FAO/WHO, 2009). As mentioned, food uses listed in Table 3.2-2 are just some of the representative target foods intended to be processed with transglutaminase. Therefore, as a conservative estimate, the proportion of solid foods that are assumed to contain the enzyme was increased to 25% for the TMDI assessment.

3.2.2.2 Theoretical Maximum Daily Intake

Based on conservative assumptions for the Budget Method described in Section 3.2.2.1 above, the TMDI of transglutaminase, from its intended use in the processing of meat products, fish products, dairy products, soybean products, baked goods and bread products, and pastas was calculated to be 1.22 mg TOS/kg body weight/day, as shown in Table 3.2.2.2-1.

Table 3.2.2.2-1 Estimated TMDI of Transglutaminase from Foods Processed with the Enzyme Intended for the General Population

Products	A Level of Consumption of Foods (kg/kg bw/day)	B Proportion of Foods Containing Transglutaminase (%)	C Level of Consumption of Foods Containing Transglutaminase (kg/kg bw/day)[a]	D Maximum Level of Transglutaminase in Foods (mg TOS/kg)	Exposure to Transglutaminase (mg TOS/kg bw/day)[b]
Solid Foods	0.05	25	0.0125	97.56	1.22

bw = body weight; TMDI = theoretical maximum daily intake; TOS = total organic solids.
[a] Calculation: (A)*(B/100).
[b] Calculation: (C)*(D).

3.2.3 Summary of the Estimated Daily Intake

The potential human exposure to transglutaminase was calculated assuming that all of the enzyme used in the processing of meat products, fish products, dairy products, soybean products, baked goods and bread products, and pastas remains in the final food. However, in most cases transglutaminase will be heat-denatured and inactivated during the final stages of processing for the final food products, which involves treatment at high temperatures, such as those that occur during food processing (*e.g.*, pasteurization or baking) or cooking by the final consumer. In cases where there is no heat treatment during processing or food preparation prior to consumption, transglutaminase may be inactivated through other factors, such as oxidation, acid or alkaline pH conditions, binding to food matrix *via* cross-linking action, and depletion of the available substrate. As such, the enzyme will not have any technological effect in final foods as consumed. A number of other conservative assumptions are also made during the exposure assessment to ensure there is no underestimation of the potential intakes of transglutaminase, including:

- Conservative assumptions made as part of the Budget Method used to calculate the TMDI (*e.g.*, increasing the proportion of solids foods assumed to contain the enzyme to 25% from the default value of 12.5% recommended by the FAO/WHO [2009]); and

- The assumption that transglutaminase is added at maximum recommended use levels and activity levels of the enzyme preparation to target foods intended for the general population at an inclusion rate of 100% (*i.e.*, all target foods are processed with transglutaminase).

Using the budget method, the TMDI of transglutaminase from the consumption of foods processed with the enzyme was estimated at 1.22 mg TOS/kg body weight/day in the general population.

Part 4. § 170.240 Self-Limiting Levels of Use

The use of transglutaminase is considered self-limiting since the substrate is protein and use levels which exceed the amount required to obtain the intended technological effect are reported to result in the decline of the organoleptic quality of the food (Ajinomoto USA, 2001).

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Transglutaminase has not been used in food before 1958.

Part 6. § 170.250 Narrative and Safety Information

6.1 Safety Narrative

The transglutaminase enzyme that is the subject of this GRAS notice is produced by submerged fermentation of a selected culture of *Streptomyces mobaraensis* M2020197. The production organism is a non-genetically modified strain of *S. mobaraensis*[3] that has a history of safe use as a source organism for transglutaminase, and has previously been concluded to be GRAS (GRN 4, 29, 55, and 95). TDS Biotech's production organism is obtained from selective culture of a wildtype *S. mobaraensis* DSM 40587[4] that is commercially available and selected based on its ability to produce high levels of transglutaminase. The identity of the production strain was confirmed by 16S rDNA sequencing to be a strain of *S. mobaraensis*.

The safety assessment of TDS Biotech's transglutaminase from *S. mobaraensis* M2020197 was conducted using the approach and principles described by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC, 1990). These guidelines are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods and have been employed in the safety assessment of enzyme preparations. The main elements that form the basis of safety of TDS Biotech's transglutaminase from *S. mobaraensis* M2020197 include information on the enzyme, source organism, manufacturing process of the enzyme, safety studies on the enzyme, as well as an estimation of dietary exposure and the resultant margin of exposure (Sewalt *et al.,* 2016). These elements are briefly summarized below and discussed in further detail in the sections that follow.

1. Transglutaminase (EC 2.3.2.13) is a well characterized enzyme that has a history of safe use as a processing aid in food production. The main enzymatic reaction of transglutaminase yields intra-molecular and inter-molecular cross-linking of proteins. The use of transglutaminase in food processing improves the texture, structure, and consistency of the final food product. TDS Biotech's transglutaminase is effectively inactivated following heat treatment at temperatures greater than 75°C (*e.g.*, cooking, baking, pasteurization) and would not have any enzymatic activity in the final food product, and therefore, exposure to the food enzyme in the final consumer is negligible.
2. The source organism was characterized and demonstrated to be non-pathogenic, non-toxigenic, and does not carry any antimicrobial resistance that would pose a safety concern. Therefore, *S. mobaraensis* M2020197 was concluded to be a suitable source organism for transglutaminase according to the Pariza and Johnson (2001) decision tree.
3. The transglutaminase enzyme is produced using food-grade materials and quality-controlled fermentation and purification/recovery processes that are common to the enzyme industry. The manufacturing process does not introduce any extraneous factor into the final food enzyme that would pose a safety concern. The transglutaminase enzyme meets the purity criteria and specifications for enzyme preparations established by JECFA and FCC.
4. Toxicology studies on a concentrate of TDS Biotech's transglutaminase from *S. mobaraensis* M2020197 (>90% TOS) demonstrate the enzyme to be non-mutagenic and non-genotoxic, and the

[3] *Streptomyces mobaraensis* was formerly known as *Streptoverticillium mobaraense* and is a well-known producer of transglutaminase used by the food industry. This species has undergone numerous safety evaluations by multiple authoritative and scientific bodies as discussed in Section 6.3.1.

[4] *Streptomyces mobaraensis* is categorized as a Biosafety Level 1 organism by the DSMZ and classified as a Group 1 biological agent that is unlikely to cause human disease according to the European Commission (Directive 2000/54/EC).

no-observed-adverse-effect level (NOAEL) to be 910 mg TOS/kg body weight/day. Based on the TMDI of 1.22 mg TOS/kg body weight/day, the resulting margin of exposure is approximately 745.

A comprehensive search of the scientific literature was conducted through May 2021 to identify publications relevant to the safety of transglutaminase. The search was performed using ProQuest Dialog™ and the following databases: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. The searched identified one publication on transglutaminase from *Bacillus circulans* (de Souza *et al.,* 2011). Information supporting the GRAS conclusions of TDS Biotech's transglutaminase from *S. mobaraensis* M2020197 is discussed in the sections below.

6.2 Safety of the Production Strain

6.2.1 History of Use of the Production Strain in Food Processing

TDS Biotech's production organism used in the production of transglutaminase is a non-genetically modified strain of *S. mobaraensis* derived from selective culture of a wildtype strain of *S. mobaraensis* DSM 40587. *S. mobaraensis*, formerly known as *Streptoverticillium mobaraense*[5], is a well-known and well-documented producer of microbial transglutaminase used in food production (Pariza and Johnson, 2001; JORF, 2006; Amfep, 2015; FSANZ, 2020). *S. mobaraensis* or *S. mobaraense* is a recognized source organism for transglutaminase, as listed in the IPA Database maintained by the Codex Committee of Food Additives (CCFA) (Codex Alimentarius, 2020) and is authorized for use in a number of global jurisdictions, including the U.S. (see Section 6.3.1 for further details). The use of transglutaminase derived from *Streptomyces* or *Streptoverticillium* species in the production of various food products is well recognized and documented in the scientific literature (Kuraishi *et al*., 2001; de Góes-Favoni and Bueno, 2014; Santhi *et al*., 2017). Thus, there exists a long history of safe use of *S. mobaraensis* in food production.

A search of the PubMed database through May 2021 did not identify any publications or case reports indicating any toxigenic or pathogenic effect of *S mobaraensis*. The genus *Streptoverticillium* has not been reported to contain any pathogenic species, and has never been implicated in food-borne toxicity (Isenberg and Painter, 1980; Bergey's Manual, 1994; reviewed in Ajinomoto USA, 1997). These findings are consistent with previous GRAS evaluations, which have concluded that *S. mobaraense* is non-toxigenic and non-pathogenic, and is a safe source organism for the production of transglutaminase. These conclusions were further corroborated by a bioinformatics analysis of the production strain in which no virulent factor, toxigenic element, or antimicrobial resistance genes were identified in the annotated genome of the *S. mobaraensis* production strain that would render it unsafe when used in the production of transglutaminase (see Section 6.2.3 for further details).

6.2.2 Mycotoxins and Other Toxic Secondary Metabolites

The transglutaminase enzyme derived from *S. mobaraensis* M2020197 was analyzed for the standard list of mycotoxins required by JECFA to be tested for all enzyme preparations [*i.e*., ochratoxin A, aflatoxins (B1, B2, G1, and G2), zearalenone, sterigmatocystin, and T-2 toxin] (JECFA, 2001). Batch analyses are provided in Section 2.6.2.2 and confirm that these compounds are not present at detectable levels in the final food enzyme. Another strain of *S. mobaraensis,* IPCR 16-22, was reported to be a spore-forming, mesophilic organism that "produces toxins and antibiotic compounds" (DSMZ, 2020). The ultra-filtered

[5] https://lpsn.dsmz.de/species/streptomyces-mobaraensis.

transglutaminase enzyme concentrate from *S. mobaraensis* M2020197 was tested in a 90-day repeated-dose oral toxicity study in rats, and the findings demonstrate that the fermentation product of the *S. mobaraensis* production strain, transglutaminase, does not elicit any systemic toxicity in rats following oral exposure at doses well in excess of the estimated dietary intakes of the enzyme (see Section 6.3).

6.2.3 Bioinformatics Analysis of the Production Strain

The genomic and plasmid DNA of TDS Biotech's *S. mobaraensis* M2020197 production strain were extracted by magnetic beads, and sequenced by Personalbio[6]. The production strain's genomic DNA and plasmid were sequenced using whole genome shotgun and next-generation sequencing using the Illumina NovaSeq platform and PacBio Sequel sequencing platform. TDS Biotech also provided the assembled genome of *S. mobaraensis* DSM 40587, a BSL1 microorganism according to the DSMZ, that is considered to be non-pathogenic and non-toxigenic. The assembled genomes were then analyzed using publicly available bioinformatics tools hosted by the Pathosystems Resource Integration Center (PATRIC) and the Center for Genomic Epidemiology[7] (CGE). The bioinformatics assessment was performed using the principles and guidelines recently described by EFSA (2018, 2019, 2021) which form the basis of safety of the production strain, demonstrating that *S. mobaraensis* M2020197 is a safe and suitable source organism for the production of transglutaminase. The results of the bioinformatics assessment are summarized in the sections that follow.

6.2.3.1 Pathogenicity

The genome assembly of the *S. mobaraensis* M2020197 production strain as well as the plasmid were annotated using the RAST tool kit from PATRIC, and the annotated genome assembly was then evaluated for virulence genes using PATRIC analysis tools, which conducts sequence homology searches of the genome against lists of putative virulence genes maintained by PATRIC and originating from three manually curated online databases: PATRIC VF[8], VFDB[9], and Victors[10]. One gene encoding for CarD-like transcriptional regulator was identified in the genome of the production strain (Table 6.2.3.1-1). No virulent factors were identified in the plasmid, suggesting that the plasmid does not contain any virulent factors that would render the production strain pathogenic. The same protein was identified in the annotated reference genome of the type strain *S. mobaraensis* DSM 40587, a BSL1 microorganism according to DSMZ, and therefore, the presence of the gene encoding for a CarD-like transcriptional regulator is not considered to be indicative of a pathogenic phenotype of the *S. mobaraensis* production strain.

Table 6.2.3.1-1 PATRIC Subcategory Analyses of the Annotated Genomes of TDS Biotech's *Streptomyces mobaraensis* M2020197 Production Strain and *Streptomyces mobaraensis* DSM 40587 Specialty Genes Categorized as Virulence Factors

TDS Biotech's *S. mobaraensis* M2020197 Production Strain		***S. mobaraensis* DSM 40587**	
Source	**No. of Hits to Homologous Genes**	**Source**	**No. of Hits to Homologous Genes**
PATRIC_VF	1 (Virulence Factor)	PATRIC_VF	2 (Virulence Factor)
		Victors	1 (Virulence Factor)

PATRIC = Pathosystems Resource Integration Center.

[6] http://www.personalbio.cn/en-us/Default.aspx
[7] http://www.genomicepidemiology.org/.
[8] https://docs.patricbrc.org/user_guides/data/data_types/specialty_genes.html.
[9] http://www.mgc.ac.cn/VFs/main.htm.
[10] http://www.phidias.us/victors/.

Additional bioinformatics analysis of the *S. mobaraensis* production strain genome and plasmid assembly for pathogenicity was conducted using PathogenFinder v.1.1, an *in silico* tool maintained by the CGE to predict pathogenicity of a microorganism. PathogenFinder utilizes a validated method for predicting the pathogenicity of novel bacteria by comparing protein families from the strain of investigation to a protein family database composed of groups of proteins or protein families associated with pathogenic or non-pathogenic organisms (Cosentino *et al*., 2013). The prediction method is unique in that the model was developed without prior analysis of the proteins in the training set; the training organisms were tagged as pathogenic or non-pathogenic and identifying protein families that were frequently identified in pathogenic or non-pathogenic bacteria. As reported by Cosentino *et al*. (2013), PathogenFinder outperforms other pathogenicity prediction models that rely on taxonomy and sequence similarity to small sets of genes known to be associated with bacterial pathogenesis. Analyses of the *S. mobaraensis* production strain genome and plasmid assembly using PathogenFinder predicted that the strain was non-pathogenic to humans.

The pathogenicity of the *S. mobaraensis* production strain was also investigated through a search of the scientific literature. The PubMed database was searched using keywords for the production strain and pathogenicity and did not reveal any animal or human study or case report that would suggest the species to be pathogenic. Therefore, based on the totality of evidence, *S. mobaraensis* M2020197 was concluded to be non-pathogenic.

6.2.3.2 Toxigenicity

A review of the genomic features (*i.e.,* defined segments of a genome that often code for proteins and RNAs) of the *S. mobaraensis* M2020197 production strain revealed three proteins with possible toxic function: death on curing protein (Doc toxin), a "possible toxin to DivIC", and a hypothetical toxin. Comparison with the genomic features of *S. mobaraensis* DSM 40857 indicated that these proteins were not unique to TDS's production strain, suggesting that the presence of the genes encoding for Doc toxin and the "possible toxin to DivIC" do not elicit an undesirable phenotype. The peptide sequence of the hypothetical toxin was searched against non-redundant protein sequences in the GenBank database and significant sequence homology (100% identity, E-value = 1x10-84, bit-score = 252 bits) was identified with a Doc toxin from a *Streptomyces* organism. Likewise, a similar BLAST search of the peptide sequences of the death on curing protein (Doc toxin) and "possible toxin to DivIC" was also conducted against non-redundant protein sequences in the GenBank database, and significant sequence homology (>99%) to Fic family protein from *Streptomyces* species, including *S. mobaraensis*, suggesting that these genes are conserved across *Streptomyces* species. Fic family proteins are involved in the catalysis of post-translational modification of proteins typically using ATP as a co-factor. Conservation of these two proteins across other *Streptomyces* species was confirmed (>80% identity) in a BLAST search of the nucleotide sequence of the genes encoding for Doc toxin and "possible toxin to DivIC" against the nucleotide collection of the GenBank database. The results of these searches suggest that the presence of the genes encoding for these three proteins are highly conserved across *Streptomyces* species and are not suggestive of a safety concern.

The potential production of mycotoxins that may pose a safety concern by the *S. mobaraensis* production strain was investigated using the ToxFinder (v.1.0) tool maintained by the CGE. This tool identifies genes involved in mycotoxin synthesis in whole genome sequences. Evaluation of the assembled genome and plasmid DNA of the *S. mobaraensis* production strain using ToxFinder indicates the absence of genes encoding for aflatoxin, patulin, citrinin, trichothecene, ergot, fumonisin, and ochratoxin. These *in silico* findings were further supported by experimental data on 3 production batches of the transglutaminase produced by *S. mobaraensis* M2020197 in which no mycotoxins or secondary metabolites of concern were detected, indicating that the final food enzyme is free of these substances (see Section 2.6.2.2).

Based on the totality of evidence from the *in silico* searches, as well as the analytical data on mycotoxins and other secondary metabolites in the final food enzyme, *S. mobaraensis* M2020197 is not expected to contain any toxigenicity potential.

6.2.3.3 Antibiotic Resistance

The annotated assembly of the *S. mobaraensis* M2020197 genome and plasmid was evaluated for antibiotic resistance factors using PATRIC analysis tools, which conducts sequence homology searches of the genome against antibiotic resistance genes maintained in the Comprehensive Antibiotic Resistance Database (CARD)[11] and National Database of Antibiotic Resistant Organisms (NDARO)[12]. A total of 45 genes with homology to putative antibiotic resistance genes were identified in the genome of *S. mobaraensis* M2020197 (Table 6.2.3.3-1). These genes were classified into the following categories: antibiotic targets in susceptible species, antibiotic target modifying enzyme or replacement protein, antibiotic inactivation enzyme, gene conferring resistance *via* absence, protein altering cell wall charge conferring antibiotic resistance, efflux pump conferring antibiotic resistance, regulator modulating expression of antibiotic resistance genes. In order to interpret the identified antibiotic resistance genes in TDS Biotech's production strain, comparisons were conducted against the annotated genome of the BSL1 organism, *S. mobaraensis* DSM 40587. None of the antibiotic resistance factors identified in *S. mobaraensis* M2020197 were unique as they were also present in the reference genome, *S. mobaraensis* DSM 40587, suggesting that *S. mobaraensis* M2020197 would not present an increased risk of antimicrobial resistance compared to the type strain.

Table 6.2.3.3-1 PATRIC Subcategory Analyses of the Annotated Genomes of TDS Biotech's *Streptomyces mobaraensis* M2020197 Production Strain and *Streptomyces mobaraensis* DSM 40587 Specialty Genes Categorized as Antibiotic Resistance Genes

TDS Biotech's *S. mobaraensis* M2020197 Production Strain		***S. mobaraensis* DSM 40587**	
Source	**No. of Hits to Homologous Genes**	**Source**	**No. of Hits to Homologous Genes**
PATRIC	45 (Antibiotic Resistance)	PATRIC	44 (Antibiotic Resistance)
CARD	2 (Antibiotic Resistance)	N/A	N/A

CARD = Comprehensive Antibiotic Resistance Database; N/A = not applicable; NDARO = National Database of Antibiotic Resistant Organisms; PATRIC = Pathosystems Resource Integration Center.

Bioinformatics analyses of the *S. mobaraensis* M2020197 genome and plasmid assembly for antibiotic resistance genes was conducted using the online *in silico* tool, ResFinder (v.4.1) maintained by the CGE. ResFinder is a web-based method for predicting antimicrobial susceptibility testing from the WGS of a microorganism (Bortolaia *et al.,* 2020). Default search parameters (≥90% identity and ≥60% minimum length) were used for both chromosomal point mutations and acquired antimicrobial resistance genes. ResFinder includes four databases containing AMR genes, chromosomal gene mutations mediating AMR, translation of genotypes into phenotypes, and species-specific panels for *in silico* antibiograms. The databases containing AMR genes and chromosomal gene mutations are manually curated and consisted of 2,690 AMR genes and 266 resistance-mediating mutations, respectively. The genotype-to-phenotype tables were prepared using additional databases consisting of 57 antimicrobial compounds and extensive literature searches. ResFinder was validated using datasets of Minimum Inhibitory Concentrations (MIC) and WGS data for clinical isolates of *Escherichia coli*, *Salmonella* spp., *Campylobacter jejuni*, *Escherichia faecalis*, and *Salmonella aureus* with ≥95% genotype/phenotype agreement for 46/51 and 25/32 of the antimicrobial/species combinations for Gram-negative and Gram-positive bacteria, respectively. No

[11] https://card.mcmaster.ca/
[12] https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/

positive alignments to potential antimicrobial resistance genes were identified in the genome assembly or plasmid (Table 6.2.3.3-2).

Table 6.2.3.3-2 *In Silico* Analysis of the Genome Assembly and Plasmid DNA from the *Streptomyces mobaraensis* Strain M2020197 Using ResFinder (v.4.1)

Antimicrobial	Class	WGS-predicted Phenotype	
		Genome	Plasmid
Amikacin	Aminoglycoside	No resistance	No resistance
Amoxicillin	Beta-lactam	No resistance	No resistance
Amoxicillin+clavulanic acid	Beta-lactam	No resistance	No resistance
Ampicillin	Beta-lactam	No resistance	No resistance
Ampicillin+clavulanic acid	Beta-lactam	No resistance	No resistance
Apramycin	Aminoglycoside	No resistance	No resistance
Arbekacin	Aminoglycoside	No resistance	No resistance
Astromicin	Aminoglycoside	No resistance	No resistance
Azithromycin	Macrolide	No resistance	No resistance
Aztreonam	Beta-lactam	No resistance	No resistance
Benzylkonium chloride	Quaternary ammonium compounds	No resistance	No resistance
Butiromycin	Aminoglycoside	No resistance	No resistance
Butirosin	Aminoglycoside	No resistance	No resistance
Carbomycin	Macrolide	No resistance	No resistance
Cefepime	Beta-lactam	No resistance	No resistance
Cefixime	Beta-lactam	No resistance	No resistance
Cefotaxime	Beta-lactam	No resistance	No resistance
Cefotaxime+clavulanic acid	Beta-lactam	No resistance	No resistance
Cefoxitin	Beta-lactam	No resistance	No resistance
Ceftazidime	Beta-lactam	No resistance	No resistance
Ceftazidime+avibactam	Beta-lactam	No resistance	No resistance
Ceftriaxone	Beta-lactam	No resistance	No resistance
Cephalothin	Beta-lactam	No resistance	No resistance
Cephalotin	Beta-lactam	No resistance	No resistance
Cetylpyridinium chloride	Quaternary ammonium compounds	No resistance	No resistance
Chloramphenicol	Phenicol	No resistance	No resistance
Chlorhexidine	Quaternary ammonium compounds	No resistance	No resistance
Ciprofloxacin	Fluoroquinolone	No resistance	No resistance
Clindamycin	Lincosamide	No resistance	No resistance
Colistin	Polymyxin	No resistance	No resistance
Dalfopristin	Streptogramin a	No resistance	No resistance
Dibekacin	Aminoglycoside	No resistance	No resistance
Doxycycline	Tetracycline	No resistance	No resistance
Ertapenem	Beta-lactam	No resistance	No resistance
Erythromycin	Macrolide	No resistance	No resistance
Ethidium bromide	Quaternary ammonium compounds	No resistance	No resistance
Florfenicol	Phenicol	No resistance	No resistance
Fluoroquinolone	Under_development	No resistance	No resistance
Formaldehyde	Aldehydes	No resistance	No resistance

Table 6.2.3.3-2 *In Silico* Analysis of the Genome Assembly and Plasmid DNA from the *Streptomyces mobaraensis* Strain M2020197 Using ResFinder (v.4.1)

Antimicrobial	Class	WGS-predicted Phenotype	
		Genome	Plasmid
Fortimicin	Aminoglycoside	No resistance	No resistance
Fosfomycin	Fosfomycin	No resistance	No resistance
Fusidic acid	Steroid antibacterial	No resistance	No resistance
Gentamicin	Aminoglycoside	No resistance	No resistance
Hydrogen peroxide	Peroxides	No resistance	No resistance
Hygromycin	Aminoglycoside	No resistance	No resistance
Imipenem	Beta-lactam	No resistance	No resistance
Isepamicin	Aminoglycoside	No resistance	No resistance
Kanamycin	Aminoglycoside	No resistance	No resistance
Kasugamycin	Aminoglycoside	No resistance	No resistance
Lincomycin	Lincosamide	No resistance	No resistance
Linezolid	Oxazolidinone	No resistance	No resistance
Lividomycin	Aminoglycoside	No resistance	No resistance
Meropenem	Beta-lactam	No resistance	No resistance
Metronidazole	Nitroimidazole	No resistance	No resistance
Minocycline	Tetracycline	No resistance	No resistance
Mupirocin	Pseudomonic acid	No resistance	No resistance
Nalidixic acid	Fluoroquinolone	No resistance	No resistance
Neomycin	Aminoglycoside	No resistance	No resistance
Netilmicin	Aminoglycoside	No resistance	No resistance
Oleandomycin	Macrolide	No resistance	No resistance
Paromomycin	Aminoglycoside	No resistance	No resistance
Penicillin	Beta-lactam	No resistance	No resistance
Piperacillin	Beta-lactam	No resistance	No resistance
Piperacillin+clavulanic acid	Beta-lactam	No resistance	No resistance
Piperacillin+tazobactam	Beta-lactam	No resistance	No resistance
Pristinamycin ia	Streptogramin b	No resistance	No resistance
Pristinamycin iia	Streptogramin a	No resistance	No resistance
Quinupristin	Streptogramin b	No resistance	No resistance
Quinupristin+dalfopristin	Streptogramin a	No resistance	No resistance
Ribostamycin	Aminoglycoside	No resistance	No resistance
Rifampicin	Rifamycin	No resistance	No resistance
Sisomicin	Aminoglycoside	No resistance	No resistance
Spectinomycin	Aminocyclitol	No resistance	No resistance
Spiramycin	Macrolide	No resistance	No resistance
Streptomycin	Aminoglycoside	No resistance	No resistance
Sulfamethoxazole	Folate pathway antagonist	No resistance	No resistance
Teicoplanin	Glycopeptide	No resistance	No resistance
Telithromycin	Macrolide	No resistance	No resistance
Temocillin	Beta-lactam	No resistance	No resistance
Tetracycline	Tetracycline	No resistance	No resistance
Tiamulin	Pleuromutilin	No resistance	No resistance

Table 6.2.3.3-2 *In Silico* Analysis of the Genome Assembly and Plasmid DNA from the *Streptomyces mobaraensis* Strain M2020197 Using ResFinder (v.4.1)

Antimicrobial	Class	WGS-predicted Phenotype	
		Genome	Plasmid
Ticarcillin	Beta-lactam	No resistance	No resistance
Ticarcillin+clavulanic acid	Beta-lactam	No resistance	No resistance
Tigecycline	Tetracycline	No resistance	No resistance
Tobramycin	Aminoglycoside	No resistance	No resistance
Trimethoprim	Folate pathway antagonist	No resistance	No resistance
Tylosin	Macrolide	No resistance	No resistance
Vancomycin	Glycopeptide	No resistance	No resistance
Virginiamycin m	Streptogramin a	No resistance	No resistance
Virginiamycin s	Streptogramin b	No resistance	No resistance

WGS = whole genome sequencing.

Antimicrobial resistance of *S. mobaraensis* M2020197 was also investigated using ResFinderFG (v.1.0) maintained by the CGE. This tool identifies antimicrobial resistance phenotype based on a functional metagenomic antibiotic resistance determinants database. The genes within this database are based on results of functional metagenomic studies that were analyzed in human fecal samples, sewage, latrines, and soils (Sommer *et al*., 2009; Fu *et al*., 2012; Moore *et al*., 2013; Forsberg *et al.*, 2014; Seemann, 2014; Pehrsson *et al*., 2016). Analysis of the *S. mobaraensis* production strain whole genome sequence and plasmid using ResFinderFG 1.0 and the default values for identity (98%) and minimum length (60%) did not yield any matches, indicating no antimicrobial resistance genes are present that would result in an undesirable phenotype.

The annotated genome of *S. mobaraensis* M2020197 was also searched using the Resistance Gene Identifier (RGI) tool (Version 5.1.1) of the Comprehensive Antibiotic Resistance Database (CARD) (Version 3.1.0) (Alcock *et al.,* 2020). CARD is a curated database of reference DNA and protein sequences implicated in bacterial antimicrobial resistance. The RGI tool is used for resistome analysis and prediction from protein or nucleotide data. Based on the *S. mobaraensis* production strain's proteome, one match to *Streptomyces cinnamoneus* EF-Tu mutants conferring resistance to elfamycin was predicted. This match shared approximately 95% identity with an E-value of $1.1x10^{-220}$ and bit-score of 759.2, suggesting that this finding is significant. Elfamycin antibiotics are inhibitors of elongation factor-Tu (EF-Tu). These are primarily used as a laboratory tool for study of EF-Tu, and their clinical use is limited due to its poor pharmacokinetic profile and solubility; elfamycin antibiotics are not used as therapeutic agents, and therefore these findings do not have any clinical significance (Prezioso *et al.,* 2017).

It should be noted that CARD and ResFinder are databases recognized and recommended by EFSA for use in the prediction of antimicrobial resistance of organisms (EFSA, 2019). While the presence of a gene sequence sharing significant similarity to *S. cinnamoneus* EF-Tu mutants conferring resistance to elfamycin was identified using the RGI tool, an antibiotic resistance phenotype was not detected using the ResFinder tool; this discrepancy is due to the fact that elfamycin was not included in the ResFinder database, possibly due to its low clinical significance. Therefore, the presence of a sequence possibly encoding for elfamycin resistance in the *S. mobaraensis* M2020197 genome was not considered to be of concern and relevant to humans, considering that this class of antibiotics are not used clinically.

S. mobaraensis has been reported to produce bleomycin, however, transglutaminase from *S. mobaraensis,* as manufactured by TDS Biotech, was demonstrated to lack antimicrobial activity based on the methodology of JECFA (see Section 2.6.1 for further details).

S. mobaraensis also has been reported to synthesize a number of antibiotic resistance factors including penicillin acylase and β-lactamase (Zhang *et al.,* 2007; Zindel *et al.,* 2016; Hindra *et al.,* 2017). Penicillin acylase is involved in the catalysis of joining/breaking of the bond between the nucleus and lateral chain of β-lactam antimicrobial products (EFSA, 2017). Thus, the presence of this enzyme may confer antimicrobial resistance to β-lactam compounds or produce β-lactam compounds. Similarly, β-lactamase is implicated in β-lactam resistance due to its ability to hydrolyze the β-lactam ring, thus conferring resistance to β-lactam antimicrobial products (Zeng and Lin, 2013). The presence of β-lactamase was identified in *S. mobaraense* and discussed in GRN 4 (U.S. FDA, 1998). It was noted that while the organism produces β-lactamase, this enzyme is heat- and pH-labile; β-lactamase is inactivated at pH 2.4 or 60°C which is similar to the thermostability of transglutaminase (see Section 2.2.2). Thus, it was concluded that while *S. mobaraense* may produce this enzyme involved in resistance to β-lactam antimicrobial products, it would be readily inactivated at temperatures utilized in food processing or would be readily inactivated by gastric acid. In addition, the potential antibiotic resistance of TDS Biotech's production strain was investigated using an *in silico* approach involving the ResFinder and ResFinderFG tools of the CGE. The findings, as summarized above, suggest that *S. mobaraensis* M2020197 does not confer resistance to several classes of antibiotics of clinical importance from human pathogens, including β-lactam products.

No antibiotic resistance genes homologous to known antibiotic resistance genes from human clinical pathogens were identified using bioinformatic approaches. Although it is known that strains of *S. mobaraensis* can produce antibiotic resistance factors such as β-lactamase and penicillin acylase, the safety of these phenotypes has been previously evaluated for transglutaminase from *S. mobaraense* S-8112 and was concluded to not be of safety concern (GRN 4). A search of the PubMed database using search terms related to "antibiotic resistance" did not reveal any publication on antibiotic resistant *Streptomyces mobaraensis*, suggesting that antibiotic resistant *S. mobaraensis* has not been documented in the scientific literature. Nevertheless, the available evidence indicates that while *S. mobaraensis* may produce compounds involved in antibiotic resistance, the likelihood of the organism to show resistance to antibiotics is low.

6.3 Safety of the Enzyme

6.3.1 Existing Authorizations for Transglutaminase

The GRAS status of transglutaminase from *S. mobaraense* was notified to the U.S. FDA and filed under GRNs 4, 29, 55, and 95 without objection (U.S. FDA, 1998, 1999, 2001, 2002). The intended use of transglutaminase includes processed cheeses, yogurt, frozen desserts, vegetable protein products and meat substitutes, processed seafood, pasta, pastries, ready-to-eat cereal products, and grain mixtures (Table 4.3.1-1). The use of transglutaminase as a food binder in meat and poultry products has been reviewed by the USDA and is considered safe for its uses as a food binder at levels up to 65 ppm (USDA/FSIS, 2017).

Table 6.3.1-1 Summary of GRAS Notifications for Transglutaminase

Substance	Intended Uses	Use Level	FDA Response	GRN No.
Transglutaminase from *Streptoverticillium mobaraense*	Processed seafood as a crosslinking agent	Levels not to exceed 65 parts per million.	No questions	4 (U.S. FDA, 1998)

Table 6.3.1-1 Summary of GRAS Notifications for Transglutaminase

Substance	Intended Uses	Use Level	FDA Response	GRN No.
Transglutaminase from *S. mobaraense*	Processed cheeses, natural hard cheeses (domestic), cream cheese, refrigerated yogurt, frozen desserts, and vegetable protein dishes/vegeburgers/meat substitutes as a crosslinking agent at varying levels	Processed cheeses at a level of 250 parts per million (ppm); natural hard cheeses (domestic) at a level of 100 ppm; cream cheese at a level of 70 ppm; refrigerated yogurt at a level of 30 ppm; frozen desserts at a level of 20 ppm; vegetable protein dishes/vegeburgers/meat substitutes at a level of 25 ppm.	No questions	29 (U.S. FDA, 1999)
Transglutaminase from *S. mobaraense*	Pasta, bread, pastries, ready to eat cereal products, pizza dough, and grain mixtures as a crosslinking agent at varying levels	Pasta products at a level of 25 parts per million (ppm); bread products at a level of 15 ppm; pastry products (cakes, pies, doughnuts, *etc*.) at a level of 20 ppm; ready to eat cereal products at a level of 45 ppm; pizza dough at a level of 20 ppm; grain mixtures (burritos, tacos, *etc*.) at a level of 25 ppm.	No questions	55 (U.S. FDA, 2001)
Transglutaminase from *S. mobaraense*	Food in general as a crosslinking agent	Lowest levels necessary to achieve the desired technical effect.	No questions	95 (U.S. FDA, 2002)

Transglutaminase derived from *S. mobaraensis* (previously known as *S. mobaraense*) is permitted for use as a processing aid, in food processing, or as a food additive in Australia and New Zealand, Canada, China, France, and Japan. The Association of Manufacturers and Formulators of Enzyme Products (AMFEP) lists transglutaminase from non-genetically modified *S. mobaraensis* for use in food processing (Amfep, 2015). A non-exhaustive list of evaluations and authorizations for transglutaminase is provided in Table 6.3.1-2. In addition to these countries, transglutaminase from *S. mobaraensis* is listed in the CCFA IPA Database as authorized for use in Argentina, Brazil, Chile, Costa Rice, Denmark, Guatemala, Indonesia, Korea, Malaysia, Mexico, Philippines, Singapore, Thailand, Uruguay, and Venezuela (Codex Alimentarius, 2020).

Table 6.3.1-2 General Non-Exhaustive List of Evaluations and Authorizations for Transglutaminase

Jurisdiction	Evaluating/Authoritative Body	Enzyme Source	Permitted Uses	Reference
Australia and New Zealand	Food Standards Australia New Zealand	*Streptomyces mobaraensis*	Processing aid	FSANZ (2020)
Canada	Health Canada	*Streptoverticillium mobaraense S-8112*	Unstandardized prepared fish products, cheese products, process cheese products, cream cheese products, frozen dairy desserts; Simulated meat products; Yogurt; Bread; Flour; Whole wheat flour; Unstandardized bakery products; Brawn; Headcheese; Meat by-product loaf; Meat loaf;	Health Canada (2020)

Table 6.3.1-2 General Non-Exhaustive List of Evaluations and Authorizations for Transglutaminase

Jurisdiction	Evaluating/Authoritative Body	Enzyme Source	Permitted Uses	Reference
			(naming the prepared meat or prepared meat by-product) with (naming the non-meat ingredients); Prepared meat; Prepared meat by-product; Preserved meat; Preserved meat by-product; Pumping pickle, cover pickle and dry cure employed in the curing of preserved meat or preserved meat by-product; Sausage; Prepared poultry meat; Prepared poultry meat by-product; Preserved poultry meat; Preserved poultry meat by-product	
China	Ministry of Health	*Streptoverticillium mobaraense*	In food processing (uses not specified)	Ministry of Health of the PRC (2011)
France	L'Agence francaise de sécurité sanitatire des aliments	*Streptomyces mobaraensis, Streptoverticillium mobaraense*	Reconstituted fish and seafood products, reconstituted meat products, cheese and specialty cheese and milk products made from pasteurized milk, snacks made from plants and protein from eggs or milk, biscuits, pastries, and bread	JORF (2006)
Japan	Ministry of Health, Labour, and Welfare	N/A	Food additive	MHLW (2014)

N/A = not applicable.

6.3.2 Toxicology Studies on TDS Biotech's Transglutaminase from *S. mobaraensis* M2020197

A series of toxicology studies were conducted with a representative batch of an enzyme concentrate of transglutaminase from *S. mobaraensis* M2020197 (Batch No. H20151114 and 20141252), manufactured as described in Section 2.4, containing a high TOS% content (>90% TOS). These unpublished studies provide corroborating evidence of safety of transglutaminase under the conditions of use described in Section 1.3. All studies were performance in accordance with appropriate OECD test guidelines (*e.g.,* 408, 471, 487) and OECD GLP. A summary of the pertinent information from these studies are presented in the sections that follow.

6.3.2.1 Subchronic Toxicity

Wistar rats (7- to 8-week-old; n=10/sex/group) were administered the active form of microbial transglutaminase by gavage at doses of 0 (control), 91 (low-dose), 273 (mid-dose), or 910 (high-dose) mg

TOS/kg body weight/day, for 90 days. Body weight and food consumption were measured weekly and observed daily for general clinical signs. All animals were subject to an ophthalmological examination and functional observation battery (FOB) at the start of the study period and on the last week. At the end of the study period the animals were fasted overnight, and blood samples were collected from the abdominal aorta for standard hematology, blood coagulation, and clinical biochemistry parameters that are consistent with those described in OECD Test Guideline 408. Urine samples also were collected for urinalysis at the start and end of the study period. At the end of the study period, all surviving animals were terminated and subject to gross necropsy. Organs and tissue samples were removed, weighed, and analyzed macroscopically and microscopically.

The authors reported 5 deaths throughout the study period. One male control animal was found dead on Day 60; the death was attributed to stress. One male and female of the high-dose group was euthanized on Day 14 and 40, respectively. In the male animal, abnormal breathing was observed from Day 10 onwards, and from Day 13, the authors reported piloerection, cyanosis, and hypothermia, as well as abnormal fluid in the lungs. The duodenum, jejunum, ileum, caecum, and colon were gas-filled, and the thymus had an abnormal dark color. Upon histopathological examination, regenerated glandular stomach mucosa was observed with minimal severity. The death was considered to be accidental and related to the dose administration. In the female animal, similar conditions were observed from Day 26 onwards, and this death was also attributed to dose administration. In the mid-dose group, 1 female was euthanized on Day 20 for welfare reasons; the direct cause of death could not be determined histologically. Another female of the mid-dose group was found dead on Day 25; no clinical symptoms were observed in this animal prior to death, and histopathological evaluation was not possible due to cannibalism.

Abnormal breathing was reported in several animals in each treatment group. This effect was reported to relate to a local irritating effect due to the test article formulation, likely due to incidental influx or regurgitation of small amounts of the test article into the trachea. These findings were corroborated by inflammation in the lung upon histopathological examination. Transient piloerection and nasal discharge were observed and were considered to be related to the local irritating effects.

A slight, but statistically significant, reduction in spontaneous activity was observed in male animals of the mid-dose group after 11 weeks and in both sexes in the high-dose group after 12 weeks. These findings were consistent upon FOB analysis. Females of the mid-dose group were also reported to sleep more and were less active after 6 weeks of treatment. The authors considered these effects to not be toxicologically relevant due to an absence of effects on other neurobehavioral parameters.

No significant changes in body weight were reported in female animals. In males of the high-dose group, body weights were significantly less than the control at the end of the first week and treatment period (~6% and 7%, respectively). No other changes in body weight were reported in the low-dose and mid-dose groups. The decrease in body weights was associated with a decrease in food consumption in the same group. The authors reported a decrease in food consumption in female animals of the mid-dose (7% decrease) and high-dose (3% decrease) groups, but these reductions did not result in a loss of body weight, unlike observed in the male animals. Due to a lack of dose-dependency, the authors attributed the decrease in food consumption in female animals to be related to the local irritating effects in the stomach due to administration of the test article.

Slight but statistically significant decrease in lymphocytes was reported in males of the high-dose group. This finding was not considered to be toxicologically relevant as they were within the historical control range for the laboratory. No other changes in hematology parameters or blood coagulation were reported in any other animal or group.

A slight but significant decrease in blood urea levels in male animals of the low-dose and high-dose group were reported. This finding was not considered to be toxicologically relevant as they were within the historical control range for the laboratory. No other changes in clinical biochemistry parameters were reported in any other animal or group.

No significant changes in any urinalysis parameter were reported in any group.

Several changes in weights of the thymus, testes, prostate, thyroid/parathyroid, uterus, and pituitary glands were reported male and female animals of each treatment group. However, these findings were not statistically significant and were only reported in 1 sex, were considered incidental in nature, not dose-dependent, and were therefore not considered to be toxicologically relevant. These findings are briefly summarized as follows.

Absolute and relative-to-body thymus weights were decreased in males of the mid-dose and high-dose group; this finding was dose-dependent but was not statistically significant. A slight but not statistically significant decrease in absolute testes weight was reported in males of the mid-dose group. This finding was attributed to 1 male animal and was considered to be incidental in nature and not related to treatment. Relative-to-body prostate weights in males of the high-dose group were higher, but not statistically significant, compared to control animals. A similar effect was reported in the absolute thyroid/parathyroid weights in males of the low-dose group. These findings were not associated with any histopathological findings and were not considered to be toxicologically relevant.

In female animals, a slight non-statistically significant increase in absolute pituitary gland weight in all treatment groups, a non-statistically significant decrease in absolute ovary weight in the low-dose group, and an increase in uterus weight in the mid-dose group were reported. These findings were not considered to be toxicologically relevant as no associated histopathological changes were observed.

Several findings in the stomach and lungs were reported upon histopathological examination. Glandular stomach mucosal necrosis, including erosive and ulcerative lesions, were reported in 1 male of the control, 1 male and 2 females of the mid-dose, and 5 males and 3 females of the high-dose groups. The incidence and severity were increased in the mid-dose and high-dose group. In these same groups, the lesions were accompanied by local hemorrhage, inflammatory cell infiltration, surface/glandular cell regeneration, and/or intestinal metaplasia. Furthermore, the histopathological findings in the stomach also included mucosal necrosis, glandular stomach inflammation and regenerated glandular stomach mucosa, and mucous neck cell hypertrophy/proliferation in the mid-dose and high-dose groups. The histopathological findings in the lungs were limited to males, not dose-related, or were within the normal background lesions of this strain. These findings were considered incidental and related to test article administration.

The histopathological findings in the stomach were considered to be a local irritative effect of the test article, due to the active enzymatic nature of the test article and not a true systemic toxic effect,. These irritative effects possibly caused the mortalities in the mid-dose and high-dose animals. The reported thymic atrophy and evidence of gastric reflux in the high-dose animals are suggestive of stress, likely induced by the irritative effects of active transglutaminase in the test article. Furthermore, the observed effects on the glandular stomach in the mid-dose and high-dose animals were likely due to bolus gavage dosing of high amounts of active transglutaminase, as similar effects were not reported in other studies with transglutaminase administrated through the diet of rats and dogs at levels up to 5% of the diet (U.S. FDA, 2001). Therefore, in the absence of other toxicological findings attributable to the test article, the reported histological changes on the stomach mucosa are not considered suggestive of a systemic effect of transglutaminase, but are likely due to expected local irritating effects of the active enzyme activity on the proteinaceous tissues within the stomach. Based on the results of this study, the NOAEL of active

transglutaminase from *S. mobaraensis* M2020197 was reported to be 100 mg/kg body weight/day, equivalent to 91 mg TOS/kg body weight, the lowest dose tested.

It should be reiterated that the test article used in this study was an enzyme concentrate of transglutaminase (TOS content approximately 91%) containing an active form of the enzyme. In reality, consumers of food products processed with transglutaminase would be exposed to the inactive form of the enzyme due to food processing steps (*e.g.,* pasteurization), other inherent factors of the food matrix (*e.g.,* naturally occurring proteolytic enzymes in meats, vegetables, *etc.*), or cooking by the final consumer. The transglutaminase was demonstrated to be effectively inactivated following processing under standard food processing conditions, with and without heat treatment steps (see Section 3.1), and therefore, the final consumer would be exposed to the inactive form of transglutaminase under the intended conditions of use. Therefore, the reported irritative effects of the enzyme on proteinaceous tissues in the mid-dose and high-dose animals would not be toxicologically relevant for the final consumer. Therefore, in the context of exposure to the inactive form of transglutaminase in the final consumer, the NOAEL of inactive transglutaminase from *S. mobarensis* M2020197 was concluded to be 1,000 mg/kg body weight/day, equivalent to 910 mg TOS/kg body weight/day, the highest dose tested, indicating the enzyme to be non-toxic.

6.3.2.2 Genotoxicity

Bacterial Reverse Mutation Test

The potential mutagenicity of the active form of transglutaminase from *S. mobaraensis* M2020197 (Lot No. 20141252) was investigated in a bacterial reverse mutation assay with *Salmonella* Typhimurium TA100, TA98, TA102, TA1535, and TA1537 in the presence and absence of metabolic activation. The study was conducted in compliance with OECD Test Guideline 471 and OECD GLP (OECD, 1997, 1998b).

A concentration range-finding test was conducted using the plate incorporation method with the following concentrations in triplicate: 3, 9, 29, 91, 288, 910, 2,275, 4,550 µg TOS/plate. The main test was conducted in duplicate using the plate incorporation and pre-incubation methods with the following concentrations: 29, 91, 288, 910, 2,275, 4,550 µg TOS/plate, respectively, with and without S9 metabolic activation. No cytotoxic effects and no increases in revertant colonies were reported in any concentration. In comparison, the positive controls significantly increased the number of revertant colonies, thus confirming the validity of the test.

The authors concluded that transglutaminase from *S. mobaraensis* M2020197 does not have mutagenic potential under the conditions of this assay.

In vitro Mammalian Micronucleus Assay

The clastogenic and aneugenic potential of the transglutaminase from *S. mobaraensis* M2020197 (Lot No. 20151114) was investigated in an *in vitro* mammalian micronucleus assay with human lymphocytes. The study was conducted in accordance with OECD Test Guideline 487 and OECD GLP (OECD, 1998b, 2016).

A concentration range-finding study was conducted with concentrations of 7, 14, 29, 57, 114, 228, 455, 910, 2,275, or 4,550 µg TOS/mL, with and without S9 metabolic activation. The main assay was conducted with 2 tests (short-term and long-term incubation) with duplicate concentrations. In the first test, human lymphocytes were incubated with the test article for 4 hours at concentrations of 0, 228, 455, 910, 1,820, 2,730, 3,640, or 4,550 µg TOS/mL, with and without S9 metabolic activation. In the second test, cells were incubated for 44 hours at concentrations of 0, 228, 455, 910, 1,820, 2,730, 3,640, or 4,550 µg TOS/mL,

without S9 metabolic activation. Ethylmethanesulfonate and colchicine served as the positive control, while the culture medium alone served as the negative control. The following concentrations were selected for microscopic analyses: 2,730, 3,640, or 4,550 µg TOS/mL, with and without S9 metabolic activation (Test 1), and 1,820, 2,730, or 4,550 µg TOS/mL without S9 metabolic activation (Test 2). At the end of the study period, slides were prepared for analysis for at least 2,000 binucleated cells per concentration and micronuclei were evaluated. Cytotoxicity of each concentration was also evaluated.

No precipitation of the test article and no cytotoxic effects were reported in any concentration. In addition, no increases in the frequency of micronucleated cells were reported at any test concentration. Therefore, under the conditions of this assay, the authors concluded that transglutaminase from *S. mobaraensis* M2020197 does not have clastogenic or aneugenic potential.

6.3.3 Toxicology Studies on Transglutaminase from Other Sources

6.3.3.1 Transglutaminase from Streptoverticillium mobaraense

Toxicology studies on transglutaminase from *Streptoverticillium mobaraense*, also referred to as microbial transglutaminase, were discussed in previous GRAS notifications (*e.g.,* GRN 4, 29, 55, 95) on the enzyme and are briefly presented herein. These studies provide corroborating evidence of safety of TDS Biotech's transglutaminase from *S. mobaraensis*.

The toxicity of the same transglutaminase from *S. mobaraense* was investigated in a 90-day dietary toxicity study in rats (U.S. FDA, 2001). Limited study details were provided, however, rats (6/sex in low-dose and mid-dose; 18/sex in control and high-dose) were provided microbial transglutaminase (active or inactive form not reported) in the diet at concentrations of 0, 0.2, 1.0, or 5.0. The NOAEL was reported to be 5% of the diet, equivalent to approximately 5,000 mg/kg body weight/day. The reported NOAEL is approximately 5-fold greater than the NOAEL determined for TDS Biotech's transglutaminase from *S. mobaraensis*. Furthermore, the acute toxicity was investigated in rats (5/sex) administered microbial transglutaminase by gavage at doses of 0, 1,000, or 2,000 mg/kg. The NOEL was reported to be greater than 2,000 mg/kg. Transglutaminase from *S. mobaraense* was negative in a bacterial reverse mutation assay, chromosomal aberration test, and *in vivo* micronucleus test in male mice.

A 28-day oral toxicity study conducted in Beagle dogs (4/sex/group) consuming 0, 0.1, 1.0, or 5.0% transglutaminase from *S. mobaraense* in the diet was discussed in GRN 95 (U.S. FDA, 2001) and is briefly discussed herein. The mean intakes of transglutaminase were 0, 48.5, 496.5, 2,410.6 mg/kg/day, respectively, for males and 0, 50.1, 501.7, 2,380.6 mg/kg/day, respectively, for females. Inactive transglutaminase was also provided in the diet at a concentration of 0.1%, equivalent to approximately 48.8 and 47.6 mg/kg/day for males and females, respectively. No significant changes in body weights, food consumption, ophthalmology, hematology, clinical chemistry, urinalysis, electrocardiogram, serum protein electrophoresis, organ weights, or histological findings were reported in any study animal. It was concluded that transglutaminase (active and inactive) was well tolerated and did not elicit any treatment-related adverse effects on any study parameter at doses up to 5% of the diet, equivalent to approximately 2,411 and 2,381 mg/kg/day in male and female dogs, respectively. Limited study details were available for the 90-day study in rats discussed in GRN 4. Nevertheless, the results of the 90-day study as discussed in GRN 4 and the 28-day study in dogs discussed in GRN 95 provide corroborating evidence of safety of microbial transglutaminase.

6.3.3.2 Transglutaminase from Bacillus Circulans

6.3.3.2.1 Repeat Dose Oral Toxicity

Transglutaminase obtained from a *Bacillus circulans* strain BL32 was subject to a series of toxicological tests (de Souza *et al.,* 2011). Male Wistar rats (10/group; 90 days old; 200 to 280 g body weight) were administered the bacterial transglutaminase (liquid preparation) by gavage at doses of 0 or 150 U/kg body weight/day. Food and water were provided *ad libitum*. Clinical signs, food consumption, and body weights were measured daily. At the end of the study period, animals were killed, and blood samples were collected for hematology and clinical chemistry analyses. Hematology parameters included red blood cell count, white blood cell count, hemoglobin concentration, hematocrit concentration, mean corpuscular volume, mean corpuscular hemoglobin concentration, and differential leukocyte count. Clinical chemistry parameters included total protein concentration, creatinine concentration, and ALT. The liver, kidney, spleen, thymus, adrenal glands, heart, lungs, and brain were removed for macroscopic and microscopic analysis.

No mortalities were reported in any group. All animal appeared to be normal and no adverse clinical observations were reported. The authors reported no changes in body weights, hematology, or clinical chemistry. In addition, no adverse findings were reported upon histopathological examination of the animals.

A significant increase in relative-to-body lung weight was reported in the bacterial transglutaminase group compared to control. Microscopic examination did not identify any adverse histological finding; no changes in cell morphology and tissue characteristics were reported to be typical of lung cells.

Based on the results of this study, the authors concluded that administration of transglutaminase at doses of 150 U/kg body weight/day did not demonstrate any significant toxicological effects. Further details on the test article were not available that would ascertain the doses into a TOS basis.

6.3.3.2.2 Genotoxicity

De Souza *et al.* (2011) investigated the cytotoxicity and genotoxicity of transglutaminase from *Bacillus circulans* strain BL32 in a cytotoxicity assay with Chinese hamster lung fibroblasts (V79 cells) and genotoxicity in an alkaline comet assay and micronucleus assay. In the cytotoxicity assay, Chinese hamster lung fibroblast cells were grown for 2 days then trypsinized and 500 cells per dish were seeded in triplicate to determine their colony forming ability. Bacterial transglutaminase was added at concentrations of 150 U per treatment. After 5 days of incubation, colonies were fixed with ethanol, stained, counted, and the cell survival relative to control was measured. In the comet assay, Chinese hamster lung fibroblast cells were incubated with the bacterial transglutaminase for 3 hours, then washed, trypsinized, and resuspended in culture medium. The cell suspension was mixed with agarose and spread onto a slide and allowed to set for 5 minutes. Cells were prepared for analysis. One hundred cells (50 cells from each replicate slide) were analyzed and scored for DNA damage. In the micronucleus assay, V79 cells were incubated with bacterial transglutaminase for 3 hours in medium, washed, 2 µg/mL of Cyt-B was added and grown for 21 hours. After the incubation period, cells were trypsinized and prepared for microscopic analysis. Micronuclei were counted in 2,000 binucleated cells. MMS was used as a positive control in all assays.

The authors reported that the bacterial transglutaminase did not cause significant increases in DNA damage in the comet assay and did not increase the incidence of binucleated cells in the micronucleus assay at concentrations up to 150 U per treatment.

6.4 Allergenicity of the Enzyme

To confirm that the transglutaminase food enzyme does not contain amino acid sequences similar to known allergens that might produce an allergenic response, a sequence homology search was conducted using the AllergenOnline database version 21 (available at http://www.allergenonline.org; updated 14 February 2021) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2021). The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. A full-length alignment search of AllergenOnline was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). No matches were identified from searching with the full amino acid sequence of transglutaminase.

A second homology search was conducted according to the approach outlined by the FAO/WHO (2001) WHO/FAO (Codex Alimentarius, 2009), and more recently by FAO/WHO (2020). In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1–80, 2–81, 3–82, *etc*.) derived from the full-length transglutaminase amino acid sequence. The 80 amino acid alignment search was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility (FAO/WHO, 2001). Using this search strategy, no matches were identified. A third homology search conducted using the exact 8-mer approach did not produce any matches.

A sequence alignment search was performed on the full FASTA sequence of transglutaminase against a database of 68 full-length proteins known to elicit celiac disease maintained by FARRP. Sequence alignments sharing identity greater than 45% over the full-length sequence, and covering at least 50% of the length, and an E-value less than $1x10^{-16}$ was considered to be share significant similarity, and thus may suggest the potential for eliciting celiac disease. The search revealed sequence alignments to alpha-gliadin from *Triticum aestivum*, with identity values ranging from 20.3 to 24.5%, and corresponding E-values of 0.093 to 0.88. These findings do not suggest that the peptide sequence of transglutaminase share similarity to proteins implicated in celiac disease.

The allergenicity of transglutaminase also was considered through a search of the available scientific literature. Pedersen *et al.* (2004) evaluated the potential allergenicity of microbial transglutaminase derived from *S. mobaraense* using the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) decision tree. The allergenicity was evaluated using *in silico* searches for amino acid sequence similarity to known allergens, pepsin resistance, and protein binding to specific serum immunoglobulin E (IgE). In the allergenicity searches, no homology to known allergens were identified down to 6 contiguous amino acids. One match to a major allergen from cod fish, Gad c1 (parvalbumin), was identified with 5 contiguous amino acids. The potential for cross-reactivity was further investigated by targeted serum screening with 22 documented cod-allergic patients. Three dilutions of microbial transglutaminase (1, 10, and 100 µg/mL). No binding between patient IgE and microbial transglutaminase was reported, indicating the lack of cross-reactivity of the enzyme with cod allergen. The authors further investigated the *in vitro* digestibility of microbial transglutaminase using the method described by Astwood *et al.* (1996). Briefly, 100 mg of microbial transglutaminase was mixed with hydrochloric acid and sodium chloride and incubated at 37°C for 3 minutes. Pepsin was added and the reaction was allowed to proceed for 2 hours at 37°C. The digestion was stopped at various time intervals (1, 5, 10 30, 60, and 120 minutes) for analysis by SDS-PAGE. Samples were also prepared for analysis by trypsin digestion (0, 24, and 48 hours). The enzyme was completely digested by pepsin within 1 minute; trypsin was unable to digest all of transglutaminase after 48 hours. The authors reported transglutaminase to be inactive within 1 minute at pH 2.5. Based on the results of this study, the authors concluded that microbial transglutaminase from *S.*

mobaraense does not have allergenicity potential in consumers, due to its high digestibility potential by pepsin.

Transglutaminase is intended for use in in the production of meat products, fish products, dairy products, soybean products, baked goods and bread products, and pastas. These food products are all subject to additional processing steps, such as pasteurization, cooking, or baking, which involve heating at high temperatures that result in denaturation and inactivation of the enzyme. As shown by Pedersen *et al.* (2004), following ingestion, the enzyme would be rapidly digested by gastric enzymes in the stomach, and would not be present as intact protein to elicit allergenic reactions. Thus, based on the *in silico* findings and the available experimental data on transglutaminase, the food enzyme itself is not expected to pose any allergenic concerns. However, considering that transglutaminase is used in food processing to produce cross-linked protein products, the enzyme has been implicated recently in the pathogenesis of celiac disease due to its functional similarity to endogenous tissue transglutaminase, which is an autoantigen of celiac disease (Torsten and Aaron, 2018; Lerner and Matthias, 2019, 2020). These publications discuss the potential public health safety risk of transglutaminase, particularly in individuals with celiac disease, as the enzyme may act on gliadin peptides, thus forming cross-linked complexes of transglutaminase and gliadin that may be immunogenic. The potential immunogenic effect of these transglutaminase complexes was recognized by the German Federal Institute for Risk Assessment (BfR) of Germany who noted that the products of the transglutaminase reaction (*i.e.*, cross-linked protein products) may be structurally similar to gluten, thus triggering immunological reactions in individuals with celiac disease (BfR, 2011). However, the BfR recognized that while a clinically relevant risk is possible for individuals with celiac disease, the food products to which the enzyme is added are typically thermally treated, resulting in the inactivation of the enzyme, thus rendering it unable to form these cross-linked products. Furthermore, the Agency stated that these risks may be mitigated by suitable labelling of food products produced using transglutaminase or containing transglutaminase. It should be noted that the publications by Torsten and Aaron (2018) and Lerner and Matthias (2019, 2020) were review articles discussing the theoretical pathogenesis of microbial transglutaminase in individuals with celiac disease considering similarity to endogenous tissue transglutaminase. However, these findings have not been substantiated in the scientific literature, and in certain cases, were refuted by clinical evidence. In a randomized, controlled clinical trial, 21 celiac disease patients were provided 100 g/day of wheat flour rusk manufactured with and without microbial transglutaminase (obtained from *Streptomyces mobaraense*) for 90 days (Marino *et al.,* 2017). The wheat flour rusk was confirmed to contain 80 ppm of gluten by ELISA. All subjects were monitored for appearance of main gastrointestinal or extra-intestinal symptoms every month. Serum anti-tissue transglutaminase and endomysium antibodies and creatinine concentration were also monitored. All subjects were subjected to upper endoscopy with biopsy to evaluate histological alterations at baseline and at 90 days. Subjects consuming the wheat flour rusk produced without microbial transglutaminase reported more severe abdominal pain and swelling, bloating, and nausea compared to individuals consuming the wheat flour rusk produced with transglutaminase. The authors reported that 1 of 7 individuals consuming the modified rusks and 4 of 7 individuals consuming the unmodified rusks presented intestinal villous atrophy at the end of the study period. Furthermore, the individuals consuming the unmodified were reported to have significantly increased mucosal damage compared to baseline. Treppiccione *et al.* (2017) demonstrated that wheat flour produced with transglutaminase elicited a lower immune response in HLA-DQ8 transgenic mice (expressing human MHC class II molecule linked with celiac disease) compared to gliadin. The findings by Marino *et al.* (2017) and Treppiccione *et al.* (2017) suggest that transglutaminase, when used in the production of food ingredients, may not have any clinical implications for celiac disease as previously reported (Torsten and Aaron, 2018; Lerner and Matthias, 2019, 2020). It should be reiterated that transglutaminase is used in as a processing aid in the production of food ingredients that are subject to processing steps, including heating that would render the enzyme inactive and therefore unable to catalyze its enzymatic reaction. Transglutaminase was demonstrated to be effectively inactivated in the food products in which it is

proposed for use. Thus, consumer's exposure to the enzyme is limited and any potential clinically relevant immunogenicity is anticipated to be negligible.

Based on the available data, transglutaminase from *S. mobaraensis* M2020197 itself is not considered to pose an allergenic risk to consumers. The fermentation media contains fish protein concentrate, which is used as a source of nutrients for the production organism; the protein is digested by the production organism during fermentation for growth, maintenance, and production of the food enzyme. Following the fermentation period, the media is removed during downstream processing and purification of the food enzyme, such that any residual fragments of the protein in the fermentation media would be difficult to quantify and characterize. Thus, any allergenic protein stemming from the fermentation media are not expected to pose any allergenic concern to consumers (ETA, 2005; Taylor and Baumert, 2013; Amfep, 2016). This position was maintained in recent scientific opinions by EFSA, in which the Agency noted that allergenic components of the fermentation media are not expected to be transferred into the food enzyme as they would be metabolized by the production organism for growth; similarly, food enzyme exposure by the final consumer is low and would not pose allergenic risk to the final consumer (EFSA CEP Panel, 2021b,c). It is noted that there have been no published reports to suggest an allergenic reaction to a component of the fermentation media (ETA, 2005). Nevertheless, 3 production batches of the transglutaminase enzyme concentrate (Lot No. 20200287, 20200289, 20200290) were analyzed for the presence of fish protein using a qualitative PCR method and confirmed the absence of this protein that may be carried over from the fermentation media.

Based on the information provided above, no evidence exists that might indicate that the transglutaminase produced by *S. mobaraensis* strain M2020197 would cross-react with known allergens. Additionally, there is no evidence from the available scientific literature or from the history of use of transglutaminase enzyme preparations suggesting allergenicity to transglutaminase itself in consumers. Furthermore, the enzyme would be denatured under the conditions of food processing, and therefore, would not have the potential to result in allergenicity in consumers of the final foods. Based on this information, the available evidence suggest that transglutaminase produced by *S. mobaraensis* strain M2020197 has a low risk of allergenicity in final consumers following its use in food processing.

6.5 Summary and Basis for GRAS Conclusions

Transglutaminase from *S. mobaraensis* M2020197 is intended for use as a processing aid in the production of meat products, fish products, dairy products, vegetable protein and soybean products, baked goods (including pastries) and bread products, pasta and noodles, grain mixtures, and ready-to-eat cereals in order to improve texture and promote protein binding. These proposed food uses are consistent with those previously described in GRAS notices pertaining to transglutaminase from *S. mobaraense* (GRNs 4, 29, 55, 95). The dietary exposure to TDS Biotech's transglutaminase based on the proposed food uses were estimated using the Budget Method with conservative assumptions. Based on these assumptions, the TMDI of transglutaminase from *S. mobaraensis* M2020197 was estimated to be 1.22 mg TOS/kg body weight/day.

The food enzyme is manufactured in accordance with HACCP and meets food-grade specifications that comply with food enzyme specifications established by JECFA and the *Food Chemicals Codex*.

The production strain *Streptomyces mobaraensis*, also known as *Streptoverticillium mobaraense*, has a long history of safe use. It has been used as a source organism for transglutaminase for decades and is a well-known and well-documented producer of microbial transglutaminase used in food production.
S. mobaraensis is a recognized source organism for transglutaminase, as listed in the CCFA's IPA Database (Codex Alimentarius, 2020). Microbial transglutaminase from *S. mobaraense* was concluded to be GRAS for

its intended use in a variety of food products, including processed cheeses, yogurt, frozen desserts, vegetable protein products and meat substitutes, processed seafood, pasta, pastries, ready-to-eat cereal products, and grain mixtures. The GRAS conclusions were filed without objection by the U.S. FDA under GRNs 4, 29, 55, and 95 (U.S. FDA, 1998, 1999, 2001, 2002).

The safety of transglutaminase from *S. mobaraensis* M2020197 is supported by its history of safe use in the U.S. and globally. The safety of the enzyme was assessed using the Pariza and Johnson (2001) decision tree and was concluded to be safe. Based on the existing scientific literature and a bioinformatics assessment, the production strain, *S. mobaraensis* M2020197, was concluded to be non-pathogenic, non-toxigenic, and lacking antibiotic susceptibility and production, and therefore, considered a suitable source for transglutaminase, consistent with previous GRAS conclusions on this species. Transglutaminase from *S. mobaraensis* M2020197 was evaluated in a series of toxicology studies that were conducted according to OECD GLP and appropriated test guidelines. Transglutaminase was demonstrated to be non-genotoxic, which was consistent with previous findings discussed in GRN 95. The NOAEL of transglutaminase was concluded to be 1,000 mg/kg body weight/day, equivalent to 910 mg TOS/kg body weight/day,, based on the results of a 90-day oral gavage toxicity study in rodents. Localized irritative effects were reported in the stomach of mid-dose and high-dose animals. However, no other toxicologically relevant findings were reported in any other study parameter that could be attributed to the test article, and therefore, the histological changes on the stomach mucosa are not suggestive of a systemic effect of transglutaminase; rather, the irritation findings may be attributable to transglutaminase's activity on proteinaceous tissues in the stomach. Similar findings were not reported in repeated-dose oral toxicity studies with rats and dogs (U.S. FDA, 2001). Considering that transglutaminase would be effectively rendered inactive following food processing (see Section 3.1), any potential exposure in final consumers of food products processed with transglutaminase would be to the inactive form of the enzyme. Therefore, the irritative effects are not considered to be relevant under the intended conditions of use of TDS Biotech's transglutaminase. The resulting margin of exposure is estimated to be approximately 745, indicating that the intended uses of transglutaminase from *S. mobaraensis* M2020197 does not pose any safety concerns in consumers. The potential allergenicity of transglutaminase produced by *S. mobaraensis* M2020197 was assessed through sequence homology searches using an online database of known allergens. No potential cross-reactivity to transglutaminase from *S. mobaraensis* M2020197 was identified when searching with the full amino acid sequence and from the 80 amino acid sequence sliding window search. The results of the *in silico* assessment of allergenicity of transglutaminase from *S. mobaraensis* M2020197 indicate the enzyme to lack allergenic potential in consumers. Several recent publications have implicated transglutaminase in the pathogenesis of celiac disease based on the enzyme's substrate properties to form cross-linked protein complexes with gliadin that may potentially share immunogenic properties with gluten. *In vitro* digestibility studies demonstrated transglutaminase to be rapidly digested by pepsin from the gastric fluid, thus following ingestion, the enzyme would not be present as intact protein to form the aforementioned cross-linked protein complexes. Furthermore, recent publications in humans and mice have demonstrated that consumption of transglutaminase-processed wheat-based products may, in fact, have a lower potential for immunogenicity in celiac disease compared to their untreated counterparts (Marino *et al.,* 2017; Treppiccione *et al.,* 2017). It is reiterated that transglutaminase, when used in food processing, is subject to heat treatment that effectively render the enzyme inactivate, and therefore, unable to catalyze its enzymatic reaction. TDS Biotech has demonstrated the enzyme to be inactivated in food products in which it is proposed for use. Therefore, exposure to transglutaminase based on its proposed food uses in the final consumer is limited, and any potential clinically relevant immunogenicity is anticipated to be negligible. Nevertheless, the potential clinically relevant risks to individuals with celiac disease may be mitigated by appropriate labelling of food products containing transglutaminase or produced using transglutaminase.

Based on the totality of evidence, TDS Biotech has concluded that transglutaminase from *S. mobaraensis* M2020197, produced using cGMP and meeting appropriate food-grade specifications, is GRAS for use as a processing in food production on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of transglutaminase, as discussed herein, and the consensus among a panel of experts qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following scientific experts: David Brusick, (Toxicology Consultant); Professor Emeritus Michael W. Pariza (University of Wisconsin-Madison); and Professor Emeritus I. Glenn Sipes (University of Arizona). The GRAS Panel independently and collectively evaluated all data and information pertaining to the safety of TDS Biotech's transglutaminase from *S. mobaraensis* M2020197, and concluded that the transglutaminase enzyme is GRAS for use in food production, as described in Section 1.3, based on scientific procedures. A summary of the data and information reviewed by the GRAS Panel, and the GRAS Panel's conclusions, are provided in Appendix A.

Part 7. § 170.255 List of Supporting Data and Information

Ajinomoto USA (1997). *GRAS Notification for Transglutaminase in Processed Seafood: Part 2: GRAS Summary*. (GRN No. 4). Produced by Washington (DC): SRA International Inc. for Washington (DC): Ajinomoto USA. Submitted to Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=4 [Jun. 22, 1998 - FDA response - no questions].

Ajinomoto USA (2001). *GRAS Notification for Transglutaminase*. (GRN No. 95). Produced by Washington (DC): SRA International Inc. for Washington (DC): Ajinomoto USA. Submitted to Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=95 [Jul. 3, 2002 - FDA response - no questions].

Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, et al. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 48(D1):D517-D525 [plus supplementary data]. DOI:10.1093/nar/gkz935.

Amfep (2015). *List of Commercial Enzymes*. Brussels, Belgium: Association of Manufacturers & Formulators of Enzyme Products (Amfep). Available at: https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf [Updated: May 2015].

Amfep (2016). *AMFEP Statement on Labelling of Substances Capable of Causing Allergies or Intolerances Present in Food Enzyme Preparations*. (December 2016). Brussels, Belgium: Association of Manufacturers & Formulators of Enzyme Products (Amfep). Available at: https://amfep.org/_library/_files/amfep-statement-on-labelling-of-substances-capable-of-causing-allergies-or-intolerances-present-in-food-enzyme-preparations.pdf.

Astwood JD, Leach JN, Fuchs RL (1996). Stability of food allergens to digestion in vitro. Nat Biotechnol 14(10):1269-1273. DOI:10.1038/nbt1096-1269.

Bergey's Manual (1994). Holt GJ, Kreig RN, Sneath HAP, Stanley TJ, Williams TS, editors. *Bergey's Manual of Determination Bacteriology, 9th edition*. Philadelpha (PA): Lippincott, Williams & Wilkins, pp. 668-675, 2492-2504. Cited In: Ajinomoto USA, 1997 [GRN 4, Part 2].

BfR (2011). *Transglutaminase in Meat Products*. (Updated BfR opinion No. 052/2011 of 30 November 2011). Berlin, Germany: Bundesinstitut für Risikobewertung (BfR). Available at: https://www.bfr.bund.de/cm/349/transglutaminase-in-meat-products.pdf.

Codex Alimentarius (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. (Codex Alimentarius). Geneva, Switz.: World Health Organization (WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

Codex Alimentarius (2020). Protein-glutamine γ-glutamyltransferase. (CAS Number: 80146-85-6). In: *IPA Database by CCFA [Database on Processing Aids]*. Rome, Italy: Codex Alimentarius. Available at: https://ipa.cfsa.net.cn/substance?task=detail&substance_id=877 [Last accessed: September 28, 2020].

Cosentino S, Voldby Larsen M, Møller Aarestrup F, Lund O (2013). PathogenFinder--distinguishing friend from foe using bacterial whole genome sequence data. PLoS One 8(10):e77302 [11pp, erratum, 8(12)]. DOI:10.1371/journal.pone.0077302.

de Góes-Favoni SP, Bueno FR (2014). Microbial transglutaminase: general characteristics and performance in food processing technology. Food Biotechnol 28(1):1-24. DOI:10.1080/08905436.2013.870076.

de Souza CFV, Venzke JG, Rosa RM, Henriques JAP, E. Dallegrave E, Flores SH, Ayub MAZ (2011). Toxicological evaluation for food applications of transglutaminase from a newly isolated Bacillus circulans BL32. Am J food technol 6(6):460-471.

DSMZ (2020). Streptomyces mobaraensis IPCR 16-22 is a spore-forming, mesophilic bacterium that builds an aerial mycelium and produces toxins and antibiotic compounds. In: *The Bacterial Diversity Metadatabase*. Braunschweig, Germany: Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen. Available at: https://bacdive.dsmz.de/strain/16163 [Last LPSN update: 10-09-2020].

EC (2000). Directive 2000/54/EC of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC). Off J Eur Communities 43(L262):21-45. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1579115863802&uri=CELEX:32000L0054.

EFSA (2017). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 6: suitability of taxonomic units notified to EFSA until March 2017. (EFSA Panel on Biological Hazards/BIOHAZ) (Question no EFSA-Q-2016-00827, adopted: 7 June 2017 by European Food Safety Authority). EFSA J 15(7):4884 [32pp]. DOI:10.2903/j.efsa.2017.4884. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4884.

ETA (2005). *ETA Position on Food Allergen Labeling of Microbially Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials*. (Position Paper September 12, 2005). Washington (DC): Enzyme Technical Association (ETA). Available at: https://www.enzymetechnicalassociation.org/wp-content/uploads/2018/10/Allergen-psn-paper-2.pdf.

FAO/WHO (2001). *Evaluation of Allergenicity of Genetically Modified Foods*. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/gmo-allergenicity/en.

FAO/WHO (2009). Chapter 6: Dietary exposure assessment of chemicals in food. In: *Principles and Methods for the Risk Assessment of Chemicals in Food*. (Environmental Health Criteria, No. 240). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS). Available at: https://apps.who.int/iris/bitstream/handle/10665/44065/WHO_EHC_240_9_eng_Chapter6.pdf;jsessionid=423E9F9EF415CF750F5FF0200E5A22B6?sequence=9.

FARRP (2013). *Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials*. (Expert Opinion Statement by Taylor SL, Baumert JL with assistance from Enzyme Technical Association/ETA). Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: https://farrp.unl.edu/4d4aa6d1-f01e-4952-a31e-a62de78189b1.pdf.

FARRP (2020). *AllergenOnline Version 20: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org [Released: February 10, 2020].

FCC (2018). Enzyme preparations. In: *Food Chemicals Codex, 11th edition*. Rockville (MD): United States Pharmacopeia Convention (USP), pp. 413-417.

Forsberg KJ, Patel S, Gibson MK, Lauber CL, Knight R, Fierer N, et al. (2014). Bacterial phylogeny structures soil resistomes across habitats. Nature 509(7502):612-616. DOI:10.1038/nature13377.

FSANZ (2020). Schedule 18. Processing aids (F2020C00710). In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ), Australian Government. Available at: https://www.legislation.gov.au/Details/F2020C00710.

Fu L, Niu B, Zhu Z, Wu S, Li W (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28(23):3150-3152 [plus supplementary data]. DOI:10.1093/bioinformatics/bts565.

Health Canada (2020). *List of Permitted Food Enzymes*. (Lists of Permitted Food Additives, No. 5). Ottawa (ON): Health Canada. Available at: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/5-enzymes.html [Date Modified: Date Modified: 2020-10-22].

Hindra DY, Teng Q, Dong L-B, Crnovčić I, Huang T, et al. (2017). Genome mining of *Streptomyces mobaraensis* DSM40847 as a bleomycin producer providing a biotechnology platform to engineer designer bleomycin analogues. Org Lett 19(6):1386-1389. DOI:10.1021/acs.orglett.7b00283. Cited In: EFSA 2017.

IFBC (1990). Chapter 4: Safety evaluation of foods and food ingredients derived from microorganisms (International Food Biotechnology Council - IFBC). Regul Toxicol Pharmacol 12(3, Part 2):S114-S128. DOI:10.1016/S0273-2300(05)80080-7.

Isenberg HD, Painter BG (1980). Indigenous and pathogenic microorganisms of humans. In: Lennette EH, Ballows A, Hausler WJ, Truant JP, editors. *Manual of Clinical Microbiology, 3rd edition*. Washington (DC): American Society for Microbiology (ASM Press), pp. 25-39. Cited In: Ajinomoto USA, 1997 [GRN 4, Part 2].

JECFA (2001). *Safety Evaluation of Certain Mycotoxins in Food*. 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Feb. 6-15, 2001, Geneva, Switz. (WHO Food Additives Series No. 47 / FAO Food and Nutrition Paper No. 74). Geneva, Switz.: World Health Organization (WHO) / International Programme on Chemical Safety (IPCS). Available at: http://www.inchem.org/documents/jecfa/jecmono/v47je01.htm.

JECFA (2006). General specifications and considerations for enzyme preparation used in food processing [prepared by the Committee at its sixty-seventh meeting (2006)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 67th JECFA Meetings, 1956-2006. (FAO JECFA Monographs, No. 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA), xxi-xxv. Available at: http://www.fao.org/docrep/009/a0691e/a0691e00.htm.

JORF (2006). Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires NOR: ECOC0600115A [Article Annexe I C, enzymes]. J Off Repub Fr (Oct.). Available at: http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=C9CD6B3BA59CBF9B2355B79A66A0FBFE.tpdjo04v_3?idSectionTA=LEGISCTA000020667473&cidTexte=JORFTEXT000000271061&dateTexte=20140314 [Version consolidée au 28 septembre 2020].

Kieliszek M, Misiewicz A (2014). Microbial transglutaminase and its application in the food industry. A review. Folia Microbiol (Praha) 59(3):241-250. DOI:10.1007/s12223-013-0287-x.

Kuraishi C, Yamazaki K, Susa Y (2001). Transglutaminase: its utilization in the food industry. Food Rev Int 17(2):221-246. DOI:10.1081/FRI-100001258.

Lerner A, Matthias T (2019). Microbial transglutaminase should be considered as an environmental inducer of celiac disease. World J Clin Cases 7(22):3912-3914. DOI:10.12998/wjcc.v7.i22.3912.

Lerner A, Matthias T (2020). Processed food additive microbial transglutaminase and its cross-linked gliadin complexes are potential public health concerns in celiac disease. Int J Mol Sci 21(3):1127 [13pp]. DOI:10.3390/ijms21031127.

Lorand L, Graham RM (2003). Transglutaminases: crosslinking enzymes with pleiotropic functions. Nature Reviews Molecular Cell Biology 4:140–156. https://doi.org/10.1038/nrm1014.

Marino M, Casale R, Borghini R, Di Nardi S, Donato G, Angeloni A, et al. (2017). The effects of modified versus unmodified wheat gluten administration in patients with celiac disease. International Immunopharmacology 47:1-8. DOI:10.1016/j.intimp.2017.03.012.

MHLW (2014). *[List of Existing Food Additives]* [Complied and published by the Ministry of Health and Welfare on April 16, 1996]. Tokyo, Japan: Ministry of Health, Labour and Welfare, Japan (MHLW) and Japan Food Chemical Research Foundation (JFCRF) [Japanese]. Available at: https://www.ffcr.or.jp/en/tenka/list-of-existing-food-additives/list-of-existing-food-additives.html [Effective from January 30, 2014].

Ministry of Health of the PRC (2011). [*Food Safety National Standard Food Additive Standard* – English (non official) Translation of Chinese]. (National Food Safety Standard, No. GB2760-2011). Beijing, People's Republic of China: Ministry of Health of the People's Republic of China (PRC) [English (non official) translation of Chinese].

Moore AM, Patel S, Forsberg KJ, Wang B, Bentley G, Razia Y, et al. (2013). Pediatric fecal microbiota harbor diverse and novel antibiotic resistance genes. PLoS One 8(11):e78822 [9pp, plus supplementary data]. DOI:10.1371/journal.pone.0078822.

OECD (1997). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [Updated & Adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/fr/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en [New Version Updated & Adopted: 26 June 2020].

OECD (1998a). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 408) [Updated & Adopted: 21 September 1998]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en;jsessionid=1m6bvwspup322.delta [New Version Updated & Adopted: 27 June 2018].

OECD (1998b). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (2016). *In Vitro* mammalian cell micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 487) [Updated & Adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264264861-en;jsessionid=58ip5ibjcf4kc.x-oecd-live-02.

Pariza MW, Foster EM (1983). Determining the safety of enzymes used in food processing. J Food Prot 46(5):453-468 [plus addendum]. DOI:10.4315/0362-028X-46.5.453.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173-186. DOI:10.1006/rtph.2001.1466.

Pedersen MH, Hansen TK, Sten E, Seguro K, Ohtsuka T, Morita A, et al. (2004). Evaluation of the potential allergenicity of the enzyme microbial transglutaminase using the 2001 FAO/WHO Decision Tree. Mol Nutr Food Res 48(6):434-440. DOI:10.1002/mnfr.200400014.

Pehrsson EC, Tsukayama P, Patel S, Mejía-Bautista M, Sosa-Soto G, Navarrete KM, et al. (2016). Interconnected microbiomes and resistomes in low-income human habitats. Nature 533(7602):212-216 [plus supplementary data]. DOI:10.1038/nature17672.

Prezioso SM, Brown NE, Goldberg JB (2017). Elfamycins: inhibitors of elongation factor-Tu. Mol Microbiol 106(1):22-34. DOI:10.1111/mmi.13750.

Santhi D, Kalaikannan A, Malairaj P, Arun Prabhu S (2017). Application of microbial transglutaminase in meat foods: a review. Crit Rev Food Sci Nutr 57(10):2071-2076. DOI:10.1080/10408398.2014.945990.

Seemann T (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics 30(14):2068-2069. DOI:10.1093/bioinformatics/btu153.

Sewalt V, Shanahan D, Gregg L, La Marta J, Carrillo R (2016). The Generally Recognized as Safe (GRAS) process for industrial microbial enzymes. Ind Biotechnol 12(5):295-302. DOI:10.1089/ind.2016.0011.

Sommer MOA, Dantas G, Church GM (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. Science 325(5944):1128-1131. DOI:10.1126/science.1176950.

Torsten M, Aaron L (2018). Microbial transglutaminase is immunogenic and potentially pathogenic in pediatric celiac disease. Front Pediatr 6:389 [7pp]. DOI:10.3389/fped.2018.00389.

Treppiccione L, Ottombrino A, Luongo D, Maurano F, Manteca A, Lombó F, et al. (2017). Development of gluten with immunomodulatory properties using mTG-active food grade supernatants from Streptomyces mobaraensis cultures. Journal of Functional Foods 34:390-397. DOI: 10.1016/j.jff.2017.05.024.

U.S. FDA (1998). *Agency Response Letter GRAS Notice No. GRN 4 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=4 [Jun. 22, 1998 - FDA response - no questions].

U.S. FDA (1999). *Agency Response Letter GRAS Notice No. GRN 29 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=29 [Dec. 29, 1999 - FDA response - no questions].

U.S. FDA (2001). *Agency Response Letter GRAS Notice No. GRN 55 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=55 [Jan. 30, 2001 - FDA response - no questions].

U.S. FDA (2002). *Agency Response Letter GRAS Notice No. GRN 95 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=95 [Jul. 3, 2002 - FDA response - no questions].

USDA/FSIS (2001). Use of transglutaminase enzyme and pork collagen as binders in certain meat and poultry products; Direct final rule (9 CFR Parts 317, 319, and 381) [Docket No. 01–016DF]. Fed Regist (US) 66(211):54912-54916. Available at: https://www.fsis.usda.gov/wps/wcm/connect/9025eeeb-a54b-4e47-9b42-244ce16a2897/01-016DF_687.pdf?MOD=AJPERES.

USDA/FSIS (2017). *Safety of Transglutaminase Enzyme (TG enzyme)*. Washington (DC): United States Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS). Available at: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/food-labeling/safety-of-transglutaminase-tg-enzyme/safety-of-tg-enzyme [Last Modified: Feb. 6, 2017].

USDA/FSIS (2020). Part 319—Definitions and standards of identity or composition. Section §319.140—Sausage. In: *U.S. Code of Federal Regulations (CFR). Title 9: Animals and Animal Products*. (Food Safety and Inspection Service, US Department of Agriculture). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.ecfr.gov/cgi-bin/retrieveECFR?gp=1&SID=3ef81a9d8b63232d3de0951a53c12af0&h=L&mc=true&n=pt9.2.319&r=PART&ty=HTML#sp9.2.319.a.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. (2012). Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67(11):2640-2644 [plus supplementary tables]. DOI:10.1093/jac/dks261.

Zeng X, Lin J (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. Front Microbiol 4:128 [9pp]. DOI:10.3389/fmicb.2013.00128.

Zhang D, Koreishi M, Imanaka H, Imamura K, Nakanishi K (2007). Cloning and characterization of penicillin V acylase from Streptomyces mobaraensis. J Biotechnol 128(4):788-800. DOI:10.1016/j.jbiotec.2006.12.017. Cited In: EFSA, 2017.

Zindel S, Ehret V, Ehret M, Hentschel M, Witt S, Krämer A, et al. (2016). Involvement of a novel class C Beta-lactamase in the transglutaminase mediated cross-linking cascade of *Streptomyces mobaraensis* DSM 40847. PLoS One 11(2):e0149145 [16pp, plus supplementary figures]. DOI:10.1371/journal.pone.0149145.

GRAS Panel Statement on the GRAS Status of Transglutaminase from *Streptomyces mobaraensis* M2020197 for Use as a Processing Aid in Food Production

03 February 2021

INTRODUCTION

A panel of independent scientists (the "GRAS Panel"), qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened by Taixing Dongsheng Bio-Tech Co., Ltd. ("TDS Biotech") to conduct a critical and comprehensive evaluation of the available pertinent data and information related to TDS Biotech's transglutaminase derived from a non-genetically modified strain of *Streptomyces mobaraensis* M2020197 when used as a processing aid in food production. The GRAS Panel determined whether the intended uses of transglutaminase derived from *S. mobaraensis* M2020197 as a processing aid in food production would be Generally Recognized as Safe (GRAS) based on scientific procedures. For purposes of the GRAS Panel's evaluation, "safe" or "safety" indicates that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2020).

The GRAS Panel consisted of the below-signed qualified scientific experts: Professor David Brusick, (Toxicology Consultant); Professor Emeritus Michael W. Pariza (University of Wisconsin-Madison); and Professor Emeritus I. Glenn Sipes (University of Arizona). The GRAS Panel was selected and convened in accordance with the United States (U.S.) Food and Drug Administration (FDA) guidance for industry on *Best Practices for Convening a GRAS Panel* (U.S. FDA, 2017). TDS Biotech ensured that all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in food safety, toxicology, and microbiology. Efforts were placed on identifying conflicts of interest or relevant "appearance issues" that could potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or appearance issues were identified. The GRAS Panel received an honorarium as compensation for their time; the honorarium provided to the GRAS Panel was not contingent upon the outcome of their deliberations.

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of all publicly available scientific data and information compiled from a comprehensive search of the scientific literature, which included all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of transglutaminase derived from *S. mobaraensis* M2020197 as summarized in a dossier titled "*Documentation Supporting the Generally Recognized as Safe (GRAS) Use of Transglutaminase from Streptomyces mobaraensis M2020197 as a Processing Aid in Food Production*" (dated 07 December 2020). The data evaluated by the GRAS Panel included information on the identity and purity of the enzyme and production organism, method of manufacture, product specifications, batch analyses and analytical data, stability data, intended conditions of use, estimated intakes based on intended conditions of use, and a summary of the available scientific information and data pertinent to the safety of transglutaminase derived from *S. mobaraensis* M2020197, including the safety of the production organism. The GRAS Panel also evaluated other publicly available information, as considered appropriate.

Following its independent and collaborative critical evaluation of the data and information, the GRAS Panel convened *via* teleconference on 03 February 2021 and unanimously concluded that the intended uses of transglutaminase derived from *S. mobaraensis* M2020197, as described herein, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice (cGMP), are GRAS based on scientific procedures. A summary of the information critically evaluated by the GRAS Panel and serving as the basis for the GRAS Panel's conclusion is presented below.

SUMMARY AND BASIS FOR GRAS

Transglutaminase catalyzes the acyl transfer reaction of a γ-carboxyamide group of a glutamine residue in the peptide chain of protein or an ε-amino group of a lysine residue in protein as an acyl receptor to form ε-(γ-Glu)-Lys bonds, resulting in intramolecular or intermolecular crosslinking of proteins. Accordingly, transglutaminase from *S. mobaraensis* M2020197 is intended for use in the processing of meat products, fish products, dairy products, vegetable protein and soybean products, baked goods (including pastries) and bread products, pasta and noodles, grain mixtures, and ready-to-eat cereals.

The GRAS Panel reviewed data related to the characterization of the source organism. The transglutaminase food enzyme is derived from a non-genetically modified strain of *Streptomyces mobaraensis* (strain M2020197). The taxonomic identity of the production strain, *S. mobaraensis*, has been confirmed using 16S rDNA sequencing. *S. mobaraensis* was previously known as *Streptoverticillium mobaraense* and has been used as a source organism for production of transglutaminase enzymes for decades. Transglutaminase from *S. mobaraense* has been concluded to be GRAS under GRNs 4, 29, 55, and 95 (U.S. FDA, 1998, 1999, 2001, 2002).

Transglutaminase from *S. mobaraensis* M2020197 is manufactured using food-grade materials using techniques commonly employed by the food enzyme industry. The transglutaminase enzyme is produced by *S. mobaraensis* M2020197 in multiple phases: slant culture, pre-culture, and main culture. The production strain is non-genetically modified and selectively cultured to produce high transglutaminase activity; cultures showing high transglutaminase activity are isolated and selected as the production strain. Following the fermentation steps, the fermentation broth is filtered, centrifuged, and then concentrated by ultrafiltration. The enzyme is precipitated from the broth, isolated, and then freeze-dried.

The GRAS Panel reviewed data on the composition of the food enzyme. Transglutaminase from *S. mobaraensis* M2020197 is manufactured as an ultra-filtered enzyme concentrate. The enzyme concentrate is composed of approximately 85% protein, 5.8% ash, and 3.1% water. The mean transglutaminase activity is 12,592 U/g or approximately 14 U/mg TOS.

The GRAS Panel reviewed the product specifications for the identity and purity of transglutaminase from *S. mobaraensis* M2020197, which comply with the purity specifications established for enzyme preparations by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the *Food Chemicals Codex* (JECFA, 2006; FCC, 2018). Data on 3 to 6 production batches of the enzyme demonstrated that the manufacturing process consistently yields a product that complies with the established product specifications. The GRAS Panel reviewed analytical data on 3 production batches of the enzyme that demonstrated the absence of mycotoxins, antibiotic activity, secondary enzyme (protease) activity, and free amino acids. The production batch data demonstrated that there were no safety concerns with respect to the residual minerals that may have been carried over from the fermentation medium, including magnesium, iron, and sodium. The GRAS Panel reviewed shelf-life stability data of transglutaminase from *S. mobaraensis* M2020197 and confirmed that the enzyme is stable when stored in aluminum bags or plastic bags for 18 months at 21±2°C and relative humidity of 58±9%. The transglutaminase food enzyme was stable at temperatures up to approximately 50°C and between pH 5.0 to 9.0. The enzyme activity gradually declined above 60°C and had no activity at 70°C, showing that the enzyme is inactivated at temperatures greater than 70°C when held for 5 minutes. Transglutaminase from *S. mobaraensis* M2020197 has optimal enzymatic activity at pH 5.0 to 8.0 and temperatures of 45 to 55°C. The optimal pH and temperature are similar to a commercially available transglutaminase enzyme preparation derived from *S. mobaraense*.

TDS Biotech's transglutaminase from *S. mobaraensis* M2020197 is proposed for use in processed meat and fish products, dairy products (including yogurt, cheese, and frozen desserts), baked goods, pastries, bread products, pasta and noodles, grain mixtures, ready-to-eat cereals, and vegetable protein products (including soybean products such as tofu and meat substitutes/alternatives). Any residual enzyme remaining in the final food product is heat-inactivated prior to consumption of the final food (*i.e.*, during food processing or cooking by the consumer), and therefore, exposure to the enzyme in consumers of the final food product is none to negligible. For food products not subjected to heat-treatment during food processing and/or cooking by the consumer prior to consumption, transglutaminase will be further inactivated by other factors, such as oxidation, acid or alkaline pH conditions, binding to food matrix *via* cross-linking action, depletion of the available substrate, or inherent factors within the food matrix (*e.g.*, naturally occurring proteolytic enzymes in meats, vegetables, *etc.*). The use of transglutaminase is considered self-limiting since the substrate is protein and use levels which exceed the amount required to obtain the intended technological effect are reported to result in the decline of the organoleptic quality of the food (Ajinomoto USA, 2001).

Transglutaminase is currently permitted for use at levels of up to 65 ppm as a binder in certain standardized meat food products in the U.S., such as fabricated steak, roast beef parboiled and steam roasted, and sausage (USDA/FSIS, 2020), in unstandardized meat products, in meat products formulated to reduce sodium or fat content, and in binding pieces of whole muscle meat to fabricate or reform cuts of meat (USDA/FSIS, 2001). TDS Biotech intends to use their transglutaminase at similar levels currently permitted for meat and seafood processing (*i.e.*, maximum of 65 ppm). The maximum recommended use levels of the enzyme preparation and maximum transglutaminase activity of the enzyme preparation for each individual food use are summarized in Table A-1. The daily intake of the enzyme was estimated using the Budget Method. It was assumed that all of the enzyme used in food processing would remain in the final food. In addition, conservative assumptions were made during the exposure assessment in order to overestimate the potential dietary intakes of transglutaminase, such as the proportion of solid foods containing the enzyme was increased from 12.5% to 25%, and the enzyme was added at the maximum recommended use levels and activity to target foods (*i.e.*, 1.5%) at an inclusion rate of 100% (*i.e.*, all target foods are processed with transglutaminase). The theoretical maximum daily intake (TMDI) of transglutaminase from *S. mobaraensis* M2020197 was estimated to be 1.22 mg TOS/kg body weight/day in the general population. The GRAS Panel noted that the estimated intake values obtained are gross

overestimations of the exposure to the food enzyme based upon the use of the conservative Budget Method (FAO/WHO, 2009).

The GRAS Panel critically evaluated the data and information characterizing the safety of the food enzyme. The safety of the enzyme for use in food production was assessed according to the safety paradigm described by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC, 1990). These guidelines are widely accepted by the scientific community and regulatory agencies, as criteria for assessing the safety of microbial enzyme preparations used in foods and have been extensively employed in the safety assessment of enzyme preparations.

The GRAS status of transglutaminase from *S. mobaraense* was notified to the U.S. FDA and filed under GRNs 4, 29, 55, and 95 without objection for use in processed cheeses, yogurt, frozen desserts, vegetable protein products and meat substitutes, processed seafood, pasta, pastries, ready-to-eat cereal products, and grain mixtures. Transglutaminase derived from *S. mobaraensis* (previously known as *S. mobaraense*) is permitted for use as a processing aid, in food processing, or as a food additive in Australia and New Zealand, Canada, China, France, and Japan.

The production strain, *S. mobaraensis* M2020197, is non-genetically modified and obtained from selective culture of a wildtype strain of *S. mobaraensis* DSM 40587. *S. mobaraensis* was previously known as *Streptoverticillium mobaraense*, and is a well-known and well-documented producer of microbial transglutaminase used in food production (Pariza and Johnson, 2001; JORF, 2006; Amfep, 2015; FSANZ, 2020). A review of the publicly available scientific literature through January 2021 did not identify any publications indicating pathogenic or toxigenic effect of *S. mobaraensis*. The genus *Streptoverticillium* has not been reported to contain any pathogenic species, and has never been implicated in food-borne toxicity (Isenberg and Painter, 1980; Bergey's Manual, 1994; reviewed in Ajinomoto USA, 1997). These findings are consistent with previous GRAS evaluations (GRNs 4, 29, 55, 95 – U.S. FDA, 1998, 1999, 2001, 2002), which have concluded that *S. mobaraense* is non-toxigenic and non-pathogenic, and is a safe source organism for the production of transglutaminase.

The genome of the production strain was assessed using publicly available bioinformatics tools for potential pathogenic, toxigenic, and antimicrobial resistance factors that may pose a concern. The results of the analysis indicate that the production strain does not contain any virulent factors or toxigenic element that would pose any increased safety risk from *S. mobaraensis* DSM 40587, a BSL1 organism and type strain of *S. mobarensis*. The GRAS Panel noted that analytical data on 3 production batches of transglutaminase from *S. mobaraensis* M2020197 demonstrate the absence of mycotoxins and secondary metabolites of safety concern in the final product. *S. mobaraensis* has been reported to produce bleomycin; however, the food enzyme was demonstrated to lack antimicrobial activity in 3 production batches according to the method described by JECFA. Furthermore, the species was reported to synthesize antibiotic resistance factors such as penicillin acylase and β-lactamase (Zhang *et al*., 2007; Zindel *et al*., 2016; Hindra *et al*., 2017). These factors are implicated in resistance to β-lactam antimicrobial products (Zeng and Lin, 2013; EFSA, 2017). The presence of β-lactamase in *S. mobaraense* was previously discussed in GRN 4 (U.S. FDA, 1998), and it was concluded that while the organism produces β-lactamase, this enzyme is heat-labile and pH-labile, and would be inactivated at pH 2.4 or 60°C which is similar to the pH stability and thermostability of transglutaminase. Therefore, the GRAS Panel concluded that while *S. mobaraense* may produce this enzyme involved in resistance to β-lactam antimicrobial products, these factors would be readily inactivated at temperatures utilized in food processing or would be readily inactivated by gastric acid. The potential antibiotic resistance of TDS Biotech's production strain was investigated using an *in silico* approach involving publicly available tools of the Center for Genomic Epidemiology. The findings indicate that *S. mobaraensis*

M2020197 does not confer resistance to several classes of antibiotics of clinical importance from human pathogens, including β-lactam products.

The GRAS Panel concluded that the production strain, *S. mobaraensis* M2020197, was derived from an organism that has an established history of safe use in the production of food enzymes and is considered to be non-pathogenic and non-toxigenic, lacks antimicrobial activity, and does not pose any antimicrobial resistance of concern. Transglutaminase from *S. mobaraensis* M2020197, as manufactured by TDS Biotech, was concluded to be safe based on evaluation using the Pariza and Johnson (2001) decision tree.

The GRAS Panel reviewed information on the safety of the food enzyme, which was supported by a standard battery of toxicology tests, including *in vitro* genotoxicity tests and a 90-day repeated-dose oral toxicity study in rats. All tests were conducted in compliance with the Organisation of Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998a) and appropriate OECD Guidelines for the Testing of Chemicals. All toxicology studies were performed with the active form of transglutaminase enzyme concentrate (TOS content approximately 91%).

The subchronic toxicity of the active form of transglutaminase derived from *S. mobaraensis* M2020197 was investigated in a 90-day repeated-dose oral toxicity study in rats. This study was conducted according to OECD Test Guideline 408 and OECD GLP (OECD, 1998a,b). Wistar rats (10/sex/group) were administered the enzyme by gavage at doses of 0 (control), 100 (low-dose), 300 (mid-dose), or 1,000 (high-dose) mg/kg body weight/day, equivalent to 0, 91, 273, or 910 mg TOS/kg body weight/day, for 90 days. The study parameters monitored during the 13-week study were consistent with OECD Test Guideline No. 408 (OECD, 1998b). Five deaths were reported throughout the study period. The death of 1 male control animal was attributed to stress. One male and 1 female of the high-dose group was euthanized on Day 14 and 40, respectively. In the male animal, abnormal breathing was observed from Day 10 onwards and piloerection, cyanosis, and hypothermia from Day 13. Abnormal fluid was also observed in the lungs, and the duodenum, jejunum, ileum, caecum, and colon were gas-filled, and the thymus had an abnormal dark color. Upon histopathological examination, regenerated glandular stomach mucosa was observed with minimal severity. The death was considered to be accidental and related to the dose administration. In the female animal, similar conditions were observed from Day 26 onwards, and this death was also attributed to dose administration. In the mid-dose group, 1 female was euthanized on Day 20 for welfare reasons; the direct cause of death could not be determined histologically. Another female of the mid-dose group was found dead on Day 25, and had been subjected to cannibalism. Abnormal breathing was reported in several animals in each treatment group. This effect was reported to relate to a local irritating effect due to the test article formulation, likely due to incidental influx or regurgitation of small amounts of the test article into the trachea. These findings were corroborated by inflammation in the lung upon histopathological examination. Transient piloerection and nasal discharge were observed and were considered to be related to the local irritating effects. A slight but statistically significant reduction in spontaneous activity was observed in males of the mid-dose group after 11 weeks and in both sexes in the high-dose group after 12 weeks. These findings were consistent upon functional observational battery (FOB) analysis. Mid-dose female animals were reportedly sleeping more and less active after 6 weeks of treatment. No significant changes in body weight were reported in any female animal or low-dose or mid-dose males. Body weights of male animals administered the high dose were significantly decreased at the end of the treatment period and was associated with a decrease in food consumption. The GRAS Panel noted several slight, but statistically significant, decreases in lymphocytes in males of the high-dose group and decrease in blood urea levels in males of the low-dose and high-dose group that were not considered to be toxicologically relevant as the magnitude of the changes were within the historical control range for the laboratory. No changes in any other hematology, clinical chemistry, blood coagulation parameters were reported. Several changes in weights of the thymus, testes, prostate, thyroid/parathyroid, uterus, and pituitary glands were

reported in male and female animals of each treatment group. However, these findings were not statistically significant and were only reported in 1 sex, were considered incidental in nature, not dose-dependent, and were therefore not considered to be toxicologically relevant. Several findings in the stomach and lungs were reported upon histopathological examination. Glandular stomach mucosal necrosis, including erosive and ulcerative lesions, were reported in 1 male of the control, 1 male and 2 females of the mid-dose, and 5 males and 3 females of the high-dose groups. The incidence and severity were increased in the mid-dose and high-dose group. In these same groups, the lesions were accompanied by local hemorrhage, inflammatory cell infiltration, surface/glandular cell regeneration, and/or intestinal metaplasia. Furthermore, the histopathological findings in the stomach also included mucosal necrosis, glandular stomach inflammation and regenerated glandular stomach mucosa, and mucous neck cell hypertrophy/proliferation in the mid-dose and high-dose groups. Inflammation of the lungs was observed in 3 male and female animals each in the high-dose group, however these findings were not considered to be dose related and within the normal background lesions of this strain, and therefore, were considered to be incidental and related to test article administration. The histopathological findings in the stomach were considered to be a local irritative effect of the test article and not a systemic toxic effect. These irritative effects most likely caused the mortalities observed in the mid-dose and high-dose animals. The reported thymic atrophy and evidence of gastric reflux in the high-dose animals are suggestive of stress, likely induced by the irritative effects of active transglutaminase in the test article. The GRAS Panel noted that the localized irritating effects in the glandular stomach in the mid-dose and high-dose groups were most likely due to bolus gavage dosing of high amounts of active transglutaminase, which acted upon proteinaceous tissues within the stomach, as similar effects were not observed with other transglutaminases administered in the diets of rats and dogs at levels up to 5% of the diet (U.S. FDA, 2001). Therefore, in the absence of toxicological findings attributable to the test article, the reported histological changes on the stomach mucosa are not considered suggestive of a systemic effect of transglutaminase, but are likely due to expected local irritating effects of the active enzyme activity on the proteinaceous tissues within the stomach. The results of this study suggest the NOAEL of the active form of the enzyme to be 100 mg/kg body weight/day, or 91 mg TOS/kg body weight/day, the lowest dose tested. However, upon review, the GRAS Panel noted that the reported irritative effects of the enzyme on proteinaceous tissues in the mid-dose and high-dose animals would not be toxicologically relevant for the final consumer, as dietary exposures to the enzyme would be to the inactive form. The GRAS Panel reviewed information demonstrating the transglutaminase to be effectively rendered inactive in various food products under the intended conditions of use, either by heat treatment steps, or inactivation of the enzyme through inherent factors of the food matrix (*e.g.,* naturally occurring proteolytic enzymes in meats, vegetables, *etc.*), or cooking by the final consumer. Therefore, in the context of exposure to the inactive form of transglutaminase in the final consumer, the GRAS Panel concluded the NOAEL of inactive transglutaminase from *S. mobaraensis* M2020197 to be 1,000 mg/kg body weight/day, equivalent to 910 mg TOS/kg body weight/day, the highest dose tested, indicating the enzyme to be non-toxic. Based on the TMDI of 1.22 mg TOS/kg bw/day, the margin of exposure to active transglutaminase from *S. mobaraensis* M2020197 administered by gavage was estimated to be approximately 745.

The mutagenic potential of the active form of transglutaminase from *S. mobaraensis* M2020197 was investigated in a bacterial reverse mutation assay with *Salmonella* Typhimurium TA100, TA98, TA102, TA1535, and TA1537 in the presence and absence of metabolic activation. The study was conducted in compliance with OECD Test Guideline 471 and OECD GLP (OECD, 1997, 1998a). No cytotoxic effects or increases in revertant colonies were reported in any of the test concentrations up to 5,000 µg/plate, equivalent to 4,550 µg TOS/plate. The GRAS Panel concluded that transglutaminase from *S. mobaraensis* M2020197 does not have mutagenic potential under the conditions of this assay.

The clastogenic and aneugenic potential of the active form of transglutaminase from *S. mobaraensis* M2020197 was investigated in an *in vitro* mammalian micronucleus assay with human lymphocytes. The study was conducted in accordance with OECD Test Guideline 487 and OECD GLP (OECD, 1998a, 201). The main assay was conducted with short-term exposure (4 hours) at concentrations up to 5,000 µg/mL, equivalent to 4,550 µg TOS/mL, with and without S9 metabolic activation, while the long-term (44 hours) continuous assay was performed at similar concentrations without S9 metabolic activation. The following concentrations were selected for microscopic analyses: 3,000, 4,000, and 5,000 µg/mL, with and without S9 metabolic activation (short-term), and 2,000, 3,000, and 5,000 µg/mL without S9 metabolic activation (long-term). Precipitation of the test article and cytotoxic effects were not observed at any test concentration, and no increases in the frequency of micronucleated cells were reported at any test concentration. The GRAS Panel concluded that transglutaminase from *S. mobaraensis* M2020197 does not have clastogenic or aneugenic potential under the conditions of this assay.

The GRAS Panel also reviewed toxicology studies on transglutaminase derived from other sources, including *Streptoverticillium mobaraense* and *Bacillus subtilis*. The toxicology studies on transglutaminase from *S. mobaraense* were previously discussed in GRN 4 and the results provide corroborating evidence of safety of TDS Biotech's transglutaminase from *S. mobaraensis*. The subchronic toxicity of transglutaminase (active or inactive form not reported) from *S. mobaraense* was investigated in a 90-day dietary toxicity study in rats (U.S. FDA, 2001). Limited study details were available; however, the GRAS Panel noted that the reported NOAEL of 5% of the diet, equivalent to approximately 5,000 mg/kg body weight/day, was approximately 5-fold greater than the NOAEL determined for TDS Biotech's transglutaminase from *S. mobaraensis*. The NOEL of transglutaminase from *S. mobaraense* was determined to be greater than 2,000 mg/kg in an acute toxicity study in rats, and the enzyme was negative in a bacterial reverse mutation assay, chromosomal aberration test, and *in vivo* micronucleus test in male mice. The same enzyme was evaluated in a 28-day toxicity study in Beagle dogs (4/sex/group) consuming 0, 0.1, 1.0, or 5.0% transglutaminase (active form) from *S. mobaraense* in the diet (GRN 95 – U.S. FDA, 2001). The mean intakes of transglutaminase were 0, 48.5, 496.5, and 2,410.6 mg/kg/day, respectively, for males and 0, 50.1, 501.7, and 2,380.6 mg/kg/day, respectively, for females. Inactive transglutaminase was also provided in the diet at a concentration of 0.1%, equivalent to approximately 48.8 and 47.6 mg/kg/day for males and females, respectively. No significant changes in body weights, food consumption, ophthalmology, hematology, clinical chemistry, urinalysis, electrocardiogram, serum protein electrophoresis, organ weights, or histological findings were reported in any study animal. It was concluded that transglutaminase (active and inactive) was well tolerated and did not elicit any treatment-related adverse effects on any study parameter at doses up to 5% of the diet, equivalent to approximately 2,411 and 2,381 mg/kg/day in male and female dogs, respectively. Although this study was not available for review by the Panel, the reported results provide corroborating evidence of safety of microbial transglutaminase. Transglutaminase from *Bacillus circulans* was investigated in a 14-day study and cytotoxicity assay with Chinese hamster lung fibroblasts (V79 cells) and genotoxicity in an alkaline comet assay and micronucleus assay (de Souza *et al*., 2011). The GRAS Panel noted that the transglutaminase used in these studies were poorly characterized and provided insufficient data to be used in the risk assessment of TDS Biotech's transglutaminase from *S. mobaraensis*.

The allergenicity potential of transglutaminase was evaluated using the bioinformatics criteria recommended by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) (FAO/WHO, 2001; Codex Alimentarius, 2009; JECFA, 2016). The amino acid sequence for the transglutaminase was compared to the amino acid sequences of known allergens in publicly available databases. No matches with greater than 35% percent identity were identified using a window of 80 amino acids, no exact matches were identified using a window of 8 amino acids, and no sequences were considered homologous with known allergens using a full sequence search with an E-value cut-off of 1.

Therefore, dietary exposure to transglutaminase from *S. mobaraensis* M2020197 is not expected to pose a risk of allergenicity.

The amino acid sequence of transglutaminase was searched against a publicly available database of proteins known to elicit celiac disease. The search revealed sequence alignments to alpha-gliadin from *Triticum aestivum*, with identity values ranging from 20.3 to 24.5%, and corresponding E-values of 0.093 to 0.88. These matches were below the identity cut-off of 45% and E-value of $1x10^{-16}$ and therefore do not suggest that the peptide sequence of transglutaminase share similarity to proteins implicated in celiac disease.

The allergenicity of transglutaminase was considered through a search of the available scientific literature. The GRAS Panel reviewed the findings of a publication by Pedersen *et al.* (2004) who evaluated the potential allergenicity of microbial transglutaminase derived from *S. mobaraense* using the FAO/WHO decision tree. The allergenicity was evaluated using *in silico* searches for amino acid sequence similarity to known allergens, pepsin resistance, and protein binding to specific serum IgE. No sequence homology to known allergens were identified to 6 contiguous amino acids; 1 match to a major allergen from cod fish, Gad c1 (parvalbumin), was identified with 5 contiguous amino acids. The authors investigated the potential for cross-reactivity in documented cod-allergic patients (N=22) and did not observe any binding between patient IgE and microbial transglutaminase, indicating the lack of cross-reactivity of the enzyme with cod allergen (Pedersen *et al*., 2004). The study authors also reported the findings of an *in vitro* digestibility study of microbial transglutaminase and noted the enzyme to be inactivated within 1 minute at pH 2.5 and completely digested by pepsin within 1 minute. The study authors concluded that microbial transglutaminase from *S. mobaraense* does not have allergenicity potential in consumers due to its high digestibility potential by pepsin.

The GRAS Panel reviewed publications related to the involvement of transglutaminase in the pathogenesis of celiac disease due to its functional similarity to endogenous tissue transglutaminase, which is an autoantigen of celiac disease (Torsten and Aaron, 2018; Lerner and Matthias, 2019, 2020). As the enzyme may act on gliadin peptides to form cross-linked complexes of transglutaminase and gliadin that may be immunogenic, the GRAS Panel noted that there may be a possible public health safety risk in individuals with celiac disease with the use of transglutaminase. However, these concerns may be mitigated as the food products to which the enzyme is added are typically thermally treated, resulting in the inactivation of the enzyme, thus rendering it unable to form these cross-linked products. Similar conclusions were made by the German Federal Institute for Risk Assessment (BfR) of Germany (BfR, 2011). The German BfR noted that labeling would inform individuals with celiac disease on the presence of transglutaminase in consumer products (BfR, 2011). The potential implications of transglutaminase in the pathogenesis of celiac disease are not considered relevant to TDS Biotech's transglutaminase produced from *S. mobaraensis* M2020197, considering that these complexes form in the presence of active transglutaminase. Clinical evidence demonstrated that food products produced with transglutaminase do not elicit any clinically relevant immunogenicity in celiac disease patients (Marino *et al.,* 2017; Treppiccione *et al.,* 2017). TDS Biotech's transglutaminase was demonstrated to be effectively inactivated in the food products in which it is proposed for use. As such, exposure to the active enzyme in the consumer of the final product is anticipated to be negligible, thereby reducing the risk to consumers with celiac disease.

The GRAS Panel reviewed production batch data that demonstrate the final food enzyme to be absent of any potential allergens originating from the fermentation media.

The GRAS Panel concluded that transglutaminase from *S. mobaraensis* has been sufficiently characterized and does not have potential for allergenicity. The food products transglutaminase is intended for use in are subject to heat treatment steps that would render the enzyme inactive, and therefore, are expected to not

pose an allergenic risk to consumers. Currently, in the U.S., meat products produced using transglutaminase must be labeled as part of the product name, and the enzyme itself must be listed on the ingredient list (USDA/FSIS, 2017). Thus, any potential allergenic risk for individuals sensitive to transglutaminase are mitigated as these individuals may self-regulate and avoid these food products.

Following its independent and collective critical evaluation of the available information related to transglutaminase from *S. mobaraensis* M2020197, the GRAS Panel unanimously concluded that the available information supports the conclusion presented on the following page.

CONCLUSION

We, the undersigned independent qualified members of the GRAS Panel, have individually and collectively critically evaluated the data and information summarized above, and other data and information that we deemed pertinent to the safety of the intended conditions of use of transglutaminase from *Streptomyces mobaraensis* M2020197. We unanimously conclude that the proposed use of transglutaminase from *Streptomyces mobaraensis* M2020197 as a processing aid in food production, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting the appropriate food-grade specifications presented in the supporting dossier, is safe and suitable.

We further unanimously conclude that the proposed use of transglutaminase from *Streptomyces mobaraensis* M2020197 as a processing aid in food production, manufactured consistent with cGMP and meeting appropriate food-grade specifications presented in the supporting dossier, is GRAS based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

9 June 2021

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REFERENCES

Ajinomoto USA (1997). *GRAS Notification for Transglutaminase in Processed Seafood: Part 2: GRAS Summary*. (GRN No. 4). Produced by Washington (DC): SRA International Inc. for Washington (DC): Ajinomoto USA. Submitted to Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=4 [Jun. 22, 1998 - FDA response - no questions].

Ajinomoto USA (2001). *GRAS Notification for Transglutaminase*. (GRN No. 95). Produced by Washington (DC): SRA International Inc. for Washington (DC): Ajinomoto USA. Submitted to Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=95 [Jul. 3, 2002 - FDA response - no questions].

Amfep (2015). *List of Commercial Enzymes*. Brussels, Belgium: Association of Manufacturers & Formulators of Enzyme Products (Amfep). Available at: https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf [Updated: May 2015].

Bergey's Manual (1994). Holt GJ, Kreig RN, Sneath HAP, Stanley TJ, Williams TS, editors. *Bergey's Manual of Determination Bacteriology, 9th edition*. Philadelphia (PA): Lippincott, Williams & Wilkins, pp. 668-675, 2492-2504. Cited In: Ajinomoto USA, 1997 [GRN 4, Part 2].

BfR (2011). *Transglutaminase in Meat Products*. (Updated BfR opinion No. 052/2011 of 30 November 2011). Berlin, Germany: Bundesinstitut für Risikobewertung (BfR). Available at: https://www.bfr.bund.de/cm/349/transglutaminase-in-meat-products.pdf.

Codex Alimentarius (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. (Codex Alimentarius). Geneva, Switz.: World Health Organization (WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

de Souza CFV, Venzke JG, Rosa RM, Henriques JAP, E. Dallegrave E, Flores SH, Ayub MAZ (2011). Toxicological evaluation for food applications of transglutaminase from a newly isolated Bacillus circulans BL32. Am J food technol 6(6):460-471.

EFSA (2017). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 6: suitability of taxonomic units notified to EFSA until March 2017. (EFSA Panel on Biological Hazards/BIOHAZ) (Question no EFSA-Q-2016-00827, adopted: 7 June 2017 by European Food Safety Authority). EFSA J 15(7):4884 [32pp]. DOI:10.2903/j.efsa.2017.4884. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4884.

FAO/WHO (2001). *Evaluation of Allergenicity of Genetically Modified Foods*. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/gmo-allergenicity/en.

FAO/WHO (2009). Chapter 6: Dietary exposure assessment of chemicals in food. In: *Principles and Methods for the Risk Assessment of Chemicals in Food*. (Environmental Health Criteria, No. 240). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS). Available at: https://apps.who.int/iris/bitstream/handle/10665/44065/WHO_EHC_240_9_eng_Chapter6.pdf;jsessionid=423E9F9EF415CF750F5FF0200E5A22B6?sequence=9.

FCC (2018). Enzyme preparations. In: *Food Chemicals Codex, 11th edition*. Rockville (MD): United States Pharmacopeia Convention (USP), pp. 413-417.

FSANZ (2020). Schedule 18. Processing aids (F2020C00710). In: *Australia New Zealand Food Standards Code*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ), Australian Government. Available at: https://www.legislation.gov.au/Details/F2020C00710.

Hindra DY, Teng Q, Dong L-B, Crnovčić I, Huang T, et al. (2017). Genome mining of *Streptomyces mobaraensis* DSM40847 as a bleomycin producer providing a biotechnology platform to engineer designer bleomycin analogues. Org Lett 19(6):1386-1389. DOI:10.1021/acs.orglett.7b00283. Cited In: EFSA 2017.

IFBC (1990). Chapter 4: Safety evaluation of foods and food ingredients derived from microorganisms (International Food Biotechnology Council - IFBC). Regul Toxicol Pharmacol 12(3, Part 2):S114-S128. DOI:10.1016/S0273-2300(05)80080-7.

Isenberg HD, Painter BG (1980). Indigenous and pathogenic microorganisms of humans. In: Lennette EH, Ballows A, Hausler WJ, Truant JP, editors. *Manual of Clinical Microbiology, 3rd edition*. Washington (DC): American Society for Microbiology (ASM Press), pp. 25-39. Cited In: Ajinomoto USA, 1997 [GRN 4, Part 2].

JECFA (2006). General specifications and considerations for enzyme preparation used in food processing [prepared by the Committee at its sixty-seventh meeting (2006)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 67th JECFA Meetings, 1956-2006. (FAO JECFA Monographs, No. 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA), xxi-xxv. Available at: http://www.fao.org/docrep/009/a0691e/a0691e00.htm.

JECFA (2016). 4.1 Potential allergenicity of enzymes: change to the number of amino acids in segments used in allergen database searches. In: *Evaluation of Certain Food Additives and Contaminants*. Eightieth report of the of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 16-25, 2015, Rome. (WHO Technical Report Series, No. 995). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), p. 9. Available at: https://apps.who.int/iris/bitstream/handle/10665/204410/9789240695405_eng.pdf;jsessionid=6444D4974590D924A754A02C58DA899D?sequence=1.

JORF (2006). Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires NOR: ECOC0600115A [Article Annexe I C, enzymes]. J Off Repub Fr (Oct.). Available at: http://www.legifrance.gouv.fr/affichTexte.do;jsessionid=C9CD6B3BA59CBF9B2355B79A66A0FBFE.tpdjo04v_3?idSectionTA=LEGISCTA000020667473&cidTexte=JORFTEXT000000271061&dateTexte=20140314 [Version consolidée au 28 septembre 2020].

Lerner A, Matthias T (2019). Microbial transglutaminase should be considered as an environmental inducer of celiac disease. World J Clin Cases 7(22):3912-3914. DOI:10.12998/wjcc.v7.i22.3912.

Lerner A, Matthias T (2020). Processed food additive microbial transglutaminase and its cross-linked gliadin complexes are potential public health concerns in celiac disease. Int J Mol Sci 21(3):1127 [13pp]. DOI:10.3390/ijms21031127.

Marino M, Casale R, Borghini R, Di Nardi S, Donato G, Angeloni A, et al. (2017). The effects of modified versus unmodified wheat gluten administration in patients with celiac disease. International Immunopharmacology 47:1-8. DOI:10.1016/j.intimp.2017.03.012.

OECD (1997). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [Updated & Adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/fr/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en [New Version Updated & Adopted: 26 June 2020].

OECD (1998a). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 408) [Updated & Adopted: 21 September 1998]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en;jsessionid=1m6bvwspup322.delta [New Version Updated & Adopted: 27 June 2018].

OECD (1998b). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (2016). *In Vitro* mammalian cell micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 487) [Updated & Adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264264861-en;jsessionid=58ip5ibjcf4kc.x-oecd-live-02.

Pariza MW, Foster EM (1983). Determining the safety of enzymes used in food processing. J Food Prot 46(5):453-468 [plus addendum]. DOI:10.4315/0362-028X-46.5.453.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173-186. DOI:10.1006/rtph.2001.1466.

Pedersen MH, Hansen TK, Sten E, Seguro K, Ohtsuka T, Morita A, et al. (2004). Evaluation of the potential allergenicity of the enzyme microbial transglutaminase using the 2001 FAO/WHO Decision Tree. Mol Nutr Food Res 48(6):434-440. DOI:10.1002/mnfr.200400014.

Torsten M, Aaron L (2018). Microbial transglutaminase is immunogenic and potentially pathogenic in pediatric celiac disease. Front Pediatr 6:389 [7pp]. DOI:10.3389/fped.2018.00389.

Treppiccione L, Ottombrino A, Luongo D, Maurano F, Manteca A, Lombó F, et al. (2017). Development of gluten with immunomodulatory properties using mTG-active food grade supernatants from Streptomyces mobaraensis cultures. Journal of Functional Foods 34:390-397. DOI: 10.1016/j.jff.2017.05.024.

U.S. FDA (1998). *Agency Response Letter GRAS Notice No. GRN 4 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=4 [Jun. 22, 1998 - FDA response - no questions].

U.S. FDA (1999). *Agency Response Letter GRAS Notice No. GRN 29 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=29 [Dec. 29, 1999 - FDA response - no questions].

U.S. FDA (2001). *Agency Response Letter GRAS Notice No. GRN 55 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=55 [Jan. 30, 2001 - FDA response - no questions].

U.S. FDA (2002). *Agency Response Letter GRAS Notice No. GRN 95 [Transglutaminase from Streptoverticillium mobaraense, Washington (DC): Ajinomoto USA]*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Premarket Approval. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=95 [Jul. 3, 2002 - FDA response - no questions].

U.S. FDA (2017). *Best Practices for Convening a GRAS Panel: Guidance for Industry: Draft Guidance*. (Docket Number: FDA-2017-D-0085, November 2017). College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Center for Veterinary Medicine (CVM). Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-best-practices-convening-gras-panel.

U.S. FDA (2020). Part 170—Food additives. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/ [current to 4-1-20, last updated 4-1-19].

USDA/FSIS (2001). Use of transglutaminase enzyme and pork collagen as binders in certain meat and poultry products; Direct final rule (9 CFR Parts 317, 319, and 381) [Docket No. 01–016DF]. Fed Regist (US) 66(211):54912-54916. Available at: https://www.fsis.usda.gov/wps/wcm/connect/9025eeeb-a54b-4e47-9b42-244ce16a2897/01-016DF_687.pdf?MOD=AJPERES.

USDA/FSIS (2017). *Safety of Transglutaminase Enzyme (TG enzyme)*. Washington (DC): United States Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS). Available at: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/food-labeling/safety-of-transglutaminase-tg-enzyme/safety-of-tg-enzyme [Last Modified: Feb. 6, 2017].

USDA/FSIS (2020). Part 319—Definitions and standards of identity or composition. Section §319.140—Sausage. In: *U.S. Code of Federal Regulations (CFR). Title 9: Animals and Animal Products*. (Food Safety and Inspection Service, US Department of Agriculture). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.ecfr.gov/cgi-bin/retrieveECFR?gp=1&SID=3ef81a9d8b63232d3de0951a53c12af0&h=L&mc=true&n=pt9.2.319&r=PART&ty=HTML#sp9.2.319.a.

Zeng X, Lin J (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. Front Microbiol 4:128 [9pp]. DOI:10.3389/fmicb.2013.00128.

Zhang D, Koreishi M, Imanaka H, Imamura K, Nakanishi K (2007). Cloning and characterization of penicillin V acylase from Streptomyces mobaraensis. J Biotechnol 128(4):788-800. DOI:10.1016/j.jbiotec.2006.12.017. Cited In: EFSA, 2017.

Zindel S, Ehret V, Ehret M, Hentschel M, Witt S, Krämer A, et al. (2016). Involvement of a novel class C Beta-lactamase in the transglutaminase mediated cross-linking cascade of *Streptomyces mobaraensis* DSM 40847. PLoS One 11(2):e0149145 [16pp, plus supplementary figures]. DOI:10.1371/journal.pone.0149145.

Table A-1 Summary of Food-Uses and Use-Levels of Transglutaminase

Food Use Category	Representative Food Uses[a]	Maximum Recommended Use Level of the Transglutaminase Enzyme Preparation (%)	Maximum Transglutaminase Activity of the Enzyme Preparation (U/g)	Maximum Use Level of Transglutaminase (mg TOS/kg food)[b,c]
Processed meat products	Fresh/cooked/smoked/dry-fermented sausages, cooked hams, meat balls, burgers, liver pate	0.3	120	26.02
Restructured meat and fish products	Beef steak, pork steak, bacon, chicken medallions, turkey medallions, salmon	1.0	90	65.04
Fish-based products	Fish burgers, scallops, shrimps, surimi	0.5	120	43.36
Dairy products	Yoghurt, natural cheese	0.05	120	4.34
	Processed cheese	1.5	90	97.56
	Cream cheese	1.0	90	65.04
	Frozen dairy dessert	0.2	120	17.34
Vegetable protein products	Tofu and soybean products	0.25	110	19.87
	Vegetable burgers, emulsified vegetable sausages	0.2	120	17.34
	Meat substitutes	0.25	120	21.69
Baked goods (including pastries) and bread products	Bread, pizza, croissant, brioche, rolls, cakes, pies, doughnuts,	0.2	120	17.34
Grain mixtures	Burritos, tacos, tortillas	0.2	120	17.34
Pastas and noodles	Pasta and noodles	0.2	120	17.34
Ready-to-eat cereals	Ready-to-eat cereals	0.5	120	43.35

TOS = total organic solids.

[a] Transglutaminase is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition.

[b] Assuming a mean transglutaminase activity of 13.837 U/mg TOS. Calculation: (g enzyme preparation/100 g food) * (U/g enzyme preparation) / 13.837 U/mg TOS * (1,000 g/1 kg).

[c] Assumes 100% of the transglutaminase from *S. mobaraensis* used as a processing aid is carried over into final foods. In reality, foods processed with the enzyme are heat-treated during the food processing (e.g., pasteurization or baking) or cooking by the final consumer, resulting in the inactivation of the enzyme (*i.e.*, the enzyme will have no technological effect in finals foods as consumed). In food products not subjected to heat-treatment during food processing and/or cooking by the consumer prior to consumption, transglutaminase will be inactivated by other factors, such as oxidation, acid or alkaline pH conditions, binding to food matrix *via* cross-linking action, depletion of the available substrate, or inherent factors within the food matrix [*e.g.,* naturally occurring proteolytic enzymes in meats, vegetables, *etc.*].

Overbey, Katie

From: Marco Marcucci <marco.marcucci@cndsfood.com>
Sent: Sunday, April 24, 2022 10:13 PM
To: Overbey, Katie
Cc: Shahrzad Tafazoli
Subject: [EXTERNAL] Re: GRN 1021 - Follow Up Question

Dear Ms. Overbey,

well received, thanks.

Functionally speaking, transglutaminase enzyme (TG) catalyzes the cross-linking reaction of proteins through the specific linkage (inter- and intra- molecular) of protein-glutamine and protein-lysine residues.
When applied in food processing, its intended technical effect depends on the specific application we are considering.

In most of the food applicatins, the TG enzymatic activity allows the texture improvement of food products, and we can define its intended technical effect as “texturizer”.
Just in few specific applications, like reformed raw meat or fish, the TG enzymatic activity allows to connect pieces of raw meat/fish together, and we can define its intended and “visibly-detectable” technical effect as ”binder”.

To briefly resume and answer your question, the intended technical effect of using TG in food application is mainly related to texture improvement ("texturizer"), although in some applications, such as in raw reformed meat/fish, it can be better describer with the term "binder". To be remarked that even defining the intended and “visibly detectable” technical effect with two terms, this does not change the fact that the TG enzyme catalyzes exactly the same reaction in both cases.

I hope I have provided a clear explanation in response to your question, and remain at your disposal for any further needs.

Best regards,

Marco Marcucci

R&D Director
Taixing Dongsheng Bio-Tech Co., Ltd.
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On Apr 23, 2022, at 1:53 AM, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Hello Mr. Marcucci,

During our review of GRAS Notice No. 001021, we noted a clarification question about the intended use of the ingredient that we request your response to.

- Please clarify the intended technical effect of the transglutaminase enzyme preparation. Specifically, will the transglutaminase enzyme be used as a binder?

You may send your response as an email reply to this message. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice. We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you in advance.

Best,

Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

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Overbey, Katie

From: Marco Marcucci <marco.marcucci@cndsfood.com>
Sent: Wednesday, July 13, 2022 4:50 AM
To: Overbey, Katie
Cc: Shahrzad Tafazoli
Subject: [EXTERNAL] Re: FDA Question for GRN 1021

Dear Ms. Overbey,

well received, thanks.

The transglutaminase enzyme preparation is intended for use as a binding agent in certain standardized and unstandardized meat and poultry products in accordance with the USDA's permitted inclusion level for transglutaminase of up to 65 ppm. In addition, the enzyme preparation is intended for use in fish-based products, excluding Siluriformes fish products.

We remain at your disposal for any further need.

BR,

Marco Marcucci

R&D Director
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On 11 Jul 2022, at 18:20, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Dear Mr. Marcucci,
During our review of GRAS Notice No. 1021, we had an additional clarification question about intended uses:

1. Please clarify if the transglutaminase enzyme preparation will be used in the production of poultry products or Siluriformes fish products.

We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you,
Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
Regulatory Review Scientist

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U.S. Food and Drug Administration
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<image001.gif>

Overbey, Katie

From: Marco Marcucci <marco.marcucci@cndsfood.com>
Sent: Thursday, August 11, 2022 1:24 AM
To: Overbey, Katie
Cc: Shahrzad Tafazoli
Subject: [EXTERNAL] Re: Additional FDA Questions - GRN 1021

Dear Ms. Overbey,

Thank you for reaching us out, we have well received your additional comments.
Please, find below our responses to your questions.

Q1. You state on p.13 that "The resulting solution containing the transglutaminase food enzyme is freeze dried and stored". Further on p. 14 you state that "Batches that do not meet the specifications for the food enzyme are not released for further processing or for the formulation of final enzyme preparations." Please clarify if the freeze-dried enzyme is further processed and/or formulated prior to use. If so, please provide details regarding the additional processing/ formulation steps used to produce the final enzyme preparation.

A1. Taixing Dongsheng Bio-Tech Co., Ltd. manufactures the transglutaminase from *S. mobaraensis* 2020197 as a dried, ultra-filtered enzyme concentrate, that serves as the basis for commercial enzyme preparations. The dried form of the enzyme is formulated and standardised prior its commercialisation and use. The additional processing/formulation steps used to produce the final transglutaminase enzyme preparations, which are sold to the market involves the pulverisation of the enzyme, the screening and the formulation/standardisation through the mixing/blending. These enzyme preparations are predominantly in powdered form, and are formulated with food-grade materials, including maltodextrin, dextrose, or lactose as carriers and in some cases sunflower oil as a coating and anti-dusting agent. For all kinds of food enzyme preparations, the food enzyme is adjusted to the desired activity, depending on the manufacturer's needs. This enzymatic activity level allows the ease and correct dosing during food processing.

Q2. Please state if the production organism is absent in the final ingredient.

A2. Taixing Dongsheng Bio-Tech Co., Ltd. has demonstrated the absence of the production organism and viable cells of the production organism in the dried, ultra-filtered enzyme concentrate at various steps of the manufacturing process (i.e., during and after ethanol precipitation and after the drying step), by means of Gram staining and cultivation methods, as well as a colony PCR method to determine the presence of transglutaminase gene (mTG). These analyses were conducted in accordance to the methodology described in the EFSA "Scientific guidance for the submission of dossiers on Food Enzymes" (Section 1.3.4.1) and the EFSA guidance document "Characterisation of microorganisms used for the production of food enzymes" (Section 2.1). The results of these analyses demonstrated that the enzyme concentrate is devoid of residual DNA or viable cells of the production organism.

We remain at your disposal for any further need.

BR,

Marco Marcucci

R&D Director
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On 9 Aug 2022, at 19:10, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Dear Mr. Marcucci,
During our review of GRAS Notice No. 001021, we noted questions that need to be addressed. Please find the questions below.

Please format your response such that each answer immediately follows the stated question. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice.

We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.
Thank you in advance for your attention to our comments.

Best,
Katie

Questions for GRN 1021

1. You state on p.13 that “The resulting solution containing the transglutaminase food enzyme is freeze dried and stored”. Further on p. 14 you state that “Batches that do not meet the specifications for the food enzyme are not released for further processing or for the formulation of final enzyme preparations.” Please clarify if the freeze-dried enzyme is further processed and/or formulated prior to use. If so, please provide details regarding the additional processing/ formulation steps used to produce the final enzyme preparation.

2. Please state if the production organism is absent in the final ingredient.

Katie Overbey, Ph.D., M.S (she/her/hers)
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Overbey, Katie

From: Marco Marcucci <marco.marcucci@cndsfood.com>
Sent: Friday, October 7, 2022 1:10 PM
To: Overbey, Katie
Subject: [EXTERNAL] Re: GRN 1021 - Additional Question

Dear Mrs. Overbey,

Please find below the response to the additional question you raised:

Q1. You state on p.4 that the transglutaminase enzyme preparation is standardized with "food-grade diluents and carriers, such as maltodextrin, lactose, or dextrose." Please indicate whether products standardized with lactose will contain milk or milk-derived proteins.

A1. We confirm that the TG preparations standardized with lactose do not contain milk or milk derived proteins.

We remain at your disposal for any further need or question.

BR,

Marco Marcucci

R&D Director
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On 6 Oct 2022, at 18:10, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Hello Mr. Marcucci,
We have an additional question for GRN 1021 that we would like you to address:

- You state on p.4 that the transglutaminase enzyme preparation is standardized with "food-grade diluents and carriers, such as maltodextrin, lactose, or dextrose." Please indicate whether products standardized with lactose will contain milk or milk-derived proteins.

Please format your response such that each answer immediately follows the stated question. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice. We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you in advance and please let me know if you have any questions.

Katie

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