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Supporting document

Risk and technical assessment – Application A1275

Transglutaminase from GM *Bacillus licheniformis* as a processing aid

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Executive summary

Novozymes Australia Pty Limited has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme transglutaminase, EC 2.3.2.13. This transglutaminase is produced from a genetically modified (GM) strain of *Bacillus licheniformis* containing the transglutaminase gene from *Streptomyces mobaraensis*.

The enzyme is intended to be used as a processing aid in the manufacture of a range of foods (baking, brewing, cereal-based baked/cooked products, cheese, fermented dairy products, dairy analogues, egg substitutes, meat and fish products, meat and fish analogues) where it catalyses the formation of a bond between glutamine and lysine. The proposed use is technologically justified for use at levels consistent with GMP.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that their enzyme meets these specifications.

No public health and safety concerns were identified in the assessment of transglutaminase produced by this GM *B. licheniformis* under the proposed use conditions. *B. licheniformis* has a long history of safe use as a production microorganism of enzyme processing aids, including several that are already permitted in the Code. The production organism is neither pathogenic nor toxigenic. Analysis of the modified production strain confirmed the presence and stability of the inserted DNA. Bioinformatics analysis indicated that the produced transglutaminase does not have substantial homology with known toxins or food allergens.

Toxicity testing of the transglutaminase enzyme showed no evidence of genotoxicity *in* vitro and the no observed adverse effect level (NOAEL) in a 90-day oral toxicity study in rats was 372 mg total organic solids (TOS)/kg body weight (bw)/day, the highest dose tested. The theoretical maximum daily intake (TMDI) of this transglutaminase for the refined dietary exposure assessment scenario selected for the risk characterisation was 2.0 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of around 200.

In the absence of any identifiable hazard, an acceptable daily intake (ADI) of ‘not specified’ is considered appropriate.

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1 Introduction

Novozymes Australia Pty Limited has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of the enzyme transglutaminase, EC 2.3.2.13. This transglutaminase is produced from a genetically modified (GM) strain of *Bacillus licheniformis* containing the transglutaminase gene from *Streptomyces mobaraensis*.

The enzyme is intended to be used as a processing aid in the manufacture of a range of foods, where it catalyses the formation of a bond between glutamine and lysine. It would be used at the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Practice (GMP).[1]

1.1 Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine whether the proposed purpose is a solely technological purpose and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used
- evaluate potential public health and safety concerns that may arise from the use of this food enzyme produced by a GM microorganism, by considering the:
  - safety and history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s)
  - safety and history of use of the production organism
  - safety of the enzyme.

2 Food technology assessment

2.1 Identity of the enzyme

Novozymes provided relevant information regarding the identity of the enzyme, and this has been verified using the IUBMB[2] enzyme nomenclature reference database (McDonald et al 2009).

Accepted IUBMB name:	protein-glutamine γ-glutamyltransferase
Systematic name:	protein-glutamine:amine γ-glutamyltransferase
Other names/common names:	transglutaminase; fibrinoligase; fibrin stabilizing factor, glutaminylpeptide γ-glutamyltransferase; R-glutaminyl-peptide:amine γ-glutamyl transferasepolyamine transglutaminase
IUBMB enzyme nomenclature:	EC 2.3.2.13
CAS number:	80146-85-6
Reaction:	protein glutamine + alkylamine = protein N^5-alkylglutamine + NH_3

[1] GMP is defined in the Standard 1.1.2—2 of the Code.

[2] International Union of Biochemistry and Molecular Biology.

2.2 Manufacturing process

2.2.1 Production of the enzyme

Enzymes produced from microorganisms are typically produced by controlled fermentation followed by removal of the production microorganism, purification and concentration of the enzyme. Final standardisation with stabilisers, preservatives, carriers, diluents, and other approved food-grade additives and ingredients is carried out after the purification and concentration steps. The formulated enzymes are referred to as enzyme preparations, which, depending upon the application in food, may be a liquid, semi-liquid or dried product. Enzyme preparations may contain either one major active enzyme that catalyses a specific reaction during food processing or two or more active enzymes that catalyse different reactions (FAO/WHO 2020).

2.2.2 Specifications for identity and purity

There are international general specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO JECFA Monographs 26 (2021)), explicitly FAO/WHO (2006) and in the Food Chemicals Codex (FCC 2022). These specifications are included in earlier publications of the primary sources listed in section S3—2 of Schedule 3 of the Code, and enzymes used as processing aids need to meet either of these specifications. In addition, under JECFA, enzyme preparations must meet the specifications criteria contained in the individual monographs. In the case of transglutaminase, there is no individual monograph.[3]

Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided the results of analysis of three different batches of their transglutaminase. Table 1 provides a comparison of the results of those analyses with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on those results, the enzyme met all relevant specifications.

Table 1` *Analysis of manufacturer's transglutaminase enzyme concentrate compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes*

Test parameters	*Applicant's test results*	*Specifications*		
		JECFA	*Food Chemicals Codex*	*The Code - section S3—4*
Lead (mg/kg)	≤0.5	≤5	≤5	≤2
Arsenic (mg/kg)	≤0.3	-	-	≤1
Cadmium (mg/kg)	≤0.05	-	-	≤1
Mercury (mg/kg)	≤0.05	-	-	≤1
Coliforms (cfu/g)	≤4	≤30	≤30	-
***Salmonella* (in 25 g)**	ND	Absent	Negative	-

[3] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/

Test parameters	Applicant's test results	Specifications		
		JECFA	Food Chemicals Codex	The Code - section S3—4
***Escherichia coli* (in 25 g)**	ND	Absent	-	-
Antimicrobial activity	ND	Absent	-	-
Production strain	ND			

cfu = colony forming units
ND = not detected

The specification for the enzyme preparation used by the manufacturer (as provided in section A.5 of the application) includes a test for the absence of the production strain.

2.3 Technological purpose

Under the current application, transglutaminase is intended for use as a processing aid in the following food processing uses: baking, brewing, cereal-based baked/cooked products, cheese, fermented dairy products, dairy analogues, egg substitutes, meat and fish products, meat and fish analogues. The applicant requested use of the enzyme at GMP levels.

The transglutaminase preparation is used as a processing aid for cross-linking the amino acids glutamine and lysine during processing of glutamine and lysine-containing foods. As stated by the applicant, in summary, transglutaminase catalyses the hydrolysis of the formation of an isopeptide bond between γ-carboxamide groups of glutamine residue side chains and the ε-amino groups of lysine residue side chains with subsequent release of ammonia. The reaction is: protein glutamine + alkylamine = protein N^5-alkylglutamine + NH_3.

The catalytic reaction of transglutaminase is ceased by depletion of the substrate protein during food processing.

For a schematic representation of the reaction catalysed by transglutaminase, refer to its record in the enzyme database BRENDA[4].

The applicant provided information on the physical and chemical properties of their enzyme preparation. Table 2 summarises this information. The enzyme is heat-denatured at a temperature of 70°C and inactivated at a pH below 4.5. Table 3 below explains how the enzyme is heat-inactivated or denatured in the food processing uses requested by the applicant.

Table 2 *Transglutaminase enzyme preparation physical/chemical properties*

Physical/chemical properties of commercial enzyme preparation	
Appearance	Liquid preparation that is light yellow in colour
Temperature range	Active at temperatures up to 70°C

[4] Information on EC 2.3.2.13 - protein-glutamine gamma-glutamyltransferase - BRENDA Enzyme Database (brenda-enzymes.org)

	Optimum 45 - 50°C
Temperature stability	The temperature stability of the transglutaminase was determined at pH 6 and 30 minutes incubation showing that the enzyme retained 40% activity up to 50°C, and that no activity remained at 60°C and above
pH range and optimum	optimum activity pH 5.5 – 7.5 at 37°C Activity within range 4.5 - 8.5

Each requested food processing use is listed in Table 3 below. A brief summary of the technological function has been prepared, using information provided by the applicant and is supported by scientific literature describing the types of reactions catalysed by transglutaminase (e.g., Damodaran et al, 2008).

Table 3 *Technological function of transglutaminase under its proposed use*

Food use	*Technological function*
Baking	The enzyme helps form a network by forming cross-links between the proteins present in the dough. The enzyme is inactivated and heat-denatured during baking or steaming.
Brewing	The enzyme can form cross-links between the proteins and peptides formed during mashing, thereby increasing their molecular weight. As a result the proteins precipitate and can be removed by filtration. If the enzyme is added during fermentation or maturation, the enzyme is denatured during the subsequent pasteurisation process.
Baked cereal-based products including pasta and noodles.	The enzyme forms cross-links between proteins leading to improved mechanical strength of the dough. The enzyme is inactivated and heat-denatured during drying, baking/boiling or steaming.
Cheese production	The enzyme is added during the coagulation step, in order to cross-link milk proteins resulting in a stronger protein network and improved water retaining capacity, resulting in a higher yield and improved texture. The enzyme is inactivated and heat-denatured by pasteurisation or due to the low pH and temperature (cold storage) environment (for the production of some cheeses).
Fermented dairy products	The enzyme is added before or during acidification where it cross-links milk proteins. The enzyme is inactivated and heat-denatured by pasteurisation or due to the low pH and temperature (cold storage) environment .
Plant-based dairy analogues including cheese, tofu and fermented dairy analogues	The enzyme functions in the same way as in cheese production or fermented products production, using plant proteins instead of dairy proteins. The enzyme functions as a processing aid where in the food for sale it has been inactivated and heat-denatured by pasteurisation or due to the low pH and temperature (cold storage) environment, or other factors related to the food matrix. For example, the enzyme may be inactivated by other means, such as by binding to a food matrix via a cross-linking action, or depletion of the available substrate.
Egg substitutes	The enzyme cross-links e.g. plant proteins in order to give the product an egg like structure. The enzyme functions as a processing aid where in the food for sale it has been inactivated and heat-denatured by pasteurisation or due to the low pH and temperature (cold storage) environment, or other factors related to the food matrix. For example, the enzyme may be inactivated by other means, such as by binding to a food matrix via a cross-linking action, or

Food use	Technological function
	depletion of the available substrate.
Meat and fish analogues	The enzyme is added during processing in order to form cross-links between the proteins. The enzyme functions as a processing aid where in the food for sale it has been inactivated and heat-denatured by pasteurisation or due to the low pH and temperature (cold storage) environment, or other factors related to the food matrix. For example, the enzyme may be inactivated by other means, such as by binding to a food matrix via a cross-linking action, or depletion of the available substrate.
Meat and fish products, including formed products.	The enzyme is added during processing in order to form cross-links between the proteins. Products include sausages, patties, fish cakes and products which are formed or joined in the semblance of a cut of meat. The enzyme functions as a processing aid where in the food for sale it has been inactivated and heat-denatured by pasteurisation or due to the low pH and temperature (cold storage) environment, or other factors related to the food matrix. For example, the enzyme may be inactivated by other means, such as by binding to a food matrix via a cross-linking action, or depletion of the available substrate.

Thus, from Table 3 above, the technological purpose of the enzyme as stated by the applicant in the above range of food processes that will utilise this enzyme is consistent with the typical function of transglutaminase. The enzyme will only function as a food processing aid in accordance with the Code where it does not perform a technological purpose in the food for sale.

The enzyme is heat-inactivated during baking or pasteurisation, or will be inactivated by other means, such as by acid or alkaline conditions, binding to a food matrix via a cross-linking action, or depletion of the available substrate. These factors can be food producer and/or process-specific and as such, reasoning for the use of the enzyme as a processing aid is determined by the producer or marketer of the food.

2.4 Allergen considerations

Novozymes provided information as confidential commercial information (CCI) about the raw materials used during the fermentation process to produce their transglutaminase enzyme. Novozymes also confirmed that analytical testing shows no detectable amounts of the relevant allergens (to the raw materials used) that are required to be declared by the Code (Standard 1.2.3 and Schedule 9) in the enzyme preparation. Based on the information provided, FSANZ considers that the allergens of concern required to be declared by the Code are highly unlikely to be present in Novozymes' final commercial enzyme preparation (a mixture of the ultra-filtered enzyme concentrate, glycerol and water when sold in liquid form). In addition, Appendix 2.1 of the application shows no detectable amounts of the allergens listed (the list includes those specified in S9—3 of the Code).

2.5 Food technology conclusion

FSANZ concludes that the use of this transglutaminase as a processing aid for use in the nominated foods is consistent with its typical function of forming an isopeptide bond between γ-carboxamide groups of glutamine residue side chains and the ε-amino groups of lysine residue side chains. The technological purpose of transglutaminase as a processing aid has been provided for the nominated foods. FSANZ concludes that the evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the

quantity and form proposed (which must be consistent with GMP), is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

The enzyme is heat-inactivated during baking or pasteurisation, or will be inactivated by other means, such as by acid or alkaline conditions, binding to a food matrix via a cross-linking action, or depletion of the available substrate. These factors can be food-producer and/or process-specific and as such justification for the use of the enzyme as a processing aid is determined by the producer or marketer of the food.

The enzyme will only function as a food processing aid in accordance with the Code where it does not perform a technological purpose in the food for sale.

There are relevant identity and purity specifications for the enzyme in the Code, and the applicant provided evidence that the enzyme meets these specifications.

3 Safety assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of this enzyme, produced by this microorganism, as a processing aid.

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1 Source microorganisms

3.1.1 Host and production organism

The host organism of the enzyme is *Bacillus licheniformis* Si3 lineage which was derived from a natural isolate of Ca63 strain. The *B. licheniformis* belongs to phylum Bacillota which was formerly phylum Firmicutes. Our assessment found the name *B. licheniformis* is validly published under the International Code of Nomenclature of Bacteria (uniport). *B. licheniformis* is a Gram positive spore-forming bacterial species of high biotechnological interest with numerous present and potential uses, including the production of bioactive compounds that are applied in a wide range of fields, such as aquaculture, agriculture, food, biomedicine, and pharmaceutical industries (Muras et al., 2021). *B. licheniformis* is considered a Class 1 Containment Agent under the National Institute of Health (NHI, 2019). This microorganism also falls under the Class 1 Containment under the European Federation of Biotechnology guidelines (Frommer et al., 1989).

While *B. licheniformis* isolates have been reported to be associated with foodborne illness from cooked meats, ice cream, cheese, raw milk, infant feed, prawns (Salkinoja-Salonen et al. 1999), the incidence of human infections and pathogenicity is rare and tends to be limited to immune-compromised individuals (Haydushka et al, 2012; Logan, 2012).
B. licheniformis is widely used to produce food-grade enzymes and other food products (Aslam et al., 2020). FSANZ has previously assessed the safety of *B. licheniformis* for a number of food processing aids (both GM and non-GM). Schedule 18 to Standard 1.3.3 of the Code currently permits the use of the following enzymes, serine proteinase (A1098), subtilisin (A1206), Alpha-amylase (A1219), and Beta-amylase (A1220) processing aids.

Molecular data provided by the applicant confirmed the identity of the production strain as *B. licheniformis*. The production organism has been genetically modified. The analysis of characteristics of three representative batches of enzyme along with the described production methodology demonstrated that culture conditions can be applied appropriately

and consistently between batches. Methodology and results confirming that the production organism is not detected in the final enzyme production were provided by the applicant. No public health and safety concerns were identified and the production organism is neither pathogenic nor toxigenic.

3.1.2 Gene donor organism

The transglutaminase enzyme encoding gene was synthesised based on the sequence from *Streptomyces mobaraensis* that is a Biosafety Level Risk Group 1 organism[5].

3.2 Characterisation of the genetic modification(s)

3.2.1 Description of the DNA to be introduced and method of transformation

The gene that encodes the transglutaminase enzyme was *in vitro* synthesised based on the sequence from *S. mobaraensis* and available in public databases. Data provided by Novozymes and analysed by FSANZ confirmed the expected transglutaminase amino acid sequence.

An expression cassette containing the transglutaminase gene was introduced into the *B. licheniformis* host strain's genome, producing the production strain. The transglutaminase gene including the ribosome binding site from *B. amyloliquefaciens* and signal peptide from *B. licheniformis* was placed under the control of an engineered *Bacillus* promoter and the terminator from *B. licheniformis*.

A vector containing the transglutaminase expression cassette was used to transform the host strain. The transglutaminase expression cassette was inserted into the host's genome at specific integration sites. The final production strain was selected based on high transglutaminase activity.

3.2.2 Characterisation of inserted DNA

Data provided by Novozymes confirmed the presence of the inserted DNA in the production strain. The applicant also provided the results of genome sequencing which confirmed the absence of antibiotic resistance genes in the production strain.

3.2.3 Genetic stability of the inserted gene

The assessment confirmed the inserted gene is integrated into the genome of the production strain and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

To provide further evidence of the stability of the introduced transglutaminase gene, the applicant provided phenotypic data from large-scale fermentation of the production strain. These data confirmed that the transglutaminase gene is expressed over multiple generations and is stable.

3.3 Safety of the transglutaminase enzyme

3.3.1 History of safe use of transglutaminase

Transglutaminase from *Streptomyces mobaraensis* is currently permitted as a processing aid in Schedule 18 of the Code. However, transglutaminase from *Bacillus licheniformis*

[5] German classification according to TRBA 466.

containing the gene for transglutaminase from *Streptomyces mobaraensis* is not permitted and does not have a history of safe use in Australia or New Zealand. *Bacillus licheniformis* is an approved host microorganism and production strain, with a long history of safe use, for a number of enzymes in Schedule 18.

Transglutaminases have been used in food processing since the 1990s and have a long history of safe use (Pariza and Foster, 1983; Pariza and Johnson, 2001). The applicant stated that transglutaminase enzyme preparations from various microbial sources are used as processing aids in a range of countries.

There are no known reports of adverse effects arising from the consumption of transglutaminase from *S. mobaraensis*, when used as a processing aid.

3.3.2 Bioinformatics concerning potential for toxicity

A Clustal alignment (ClustalW 2.0.10)[6] was performed by the applicant comparing the transglutaminase produced by *Bacillus licheniformis* sequence against all sequences in the Uniprot[7] database (release: 2021-02-15) that contained the search word 'toxin' and not 'fragment'. There were no toxin sequences identified with a sequence similarity to the enzyme above 20% identity, indicating that the transglutaminase enzyme has no substantial homology to any known toxin sequence.

3.3.3 Toxicology data

Toxicity studies conducted with the transglutaminase that is the subject of this application include a 13-week repeated-dose dietary study in rats, and two genotoxicity studies; a bacterial reverse mutation assay (Ames test) and an *in vitro* micronucleus assay.

Animal studies

Toxicity study by dietary administration to Han Wistar rats for 13 weeks (Labcorp, 2022). Regulatory Status: GLP; conducted according to OECD TG 408

Transglutaminase (Batch PPN81685) was administered to Han Wistar (RccHan WIST strain) rats (10/sex/group) at concentrations of 0, 1875, 3750 or 5625 ppm (in terms of total organic solids (TOS)) by dietary administration *ad libitum* for 13 weeks. A control group received the basal diet containing water. The rats were group housed under standard laboratory conditions of environment and husbandry.

Animals were observed twice daily for clinical condition and fluid intake. Body weight, food consumption and detailed physical examinations for signs of toxicity were recorded weekly. Sensory reactivity, grip strength, and motor activity assessments were performed on all animals in week 12. Ophthalmic examination was conducted on all animals prior to treatment and on control and high dose animals in week 12. At the end of the treatment period, blood samples were collected from all animals for haematology and clinical chemistry analysis. All females were assessed for stage of oestrous cycle at study termination. All animals underwent a detailed necropsy at study termination, including full macroscopic examination of an extensive range of organs and tissues. Histopathological examination was conducted on the control and high-dose test groups.

One male from the high-dose test group (5625 ppm) was killed in week 10 for welfare reasons, after displaying a blood-stained nose, decreased activity, general body pallor, pale

[6] Clustal W2: http://www.clustal.org/clustal2
[7] UniProt database: https://www.uniprot.org

and partially closed eyes, piloerection and grinding teeth. Macroscopic and microscopic findings on this animal could not explain the cause of death; and it was concluded that the death of this animal was not attributed to treatment. The appearance and behaviour of all other animals were unaffected by treatment.

The overall mean achieved doses were 122, 244, and 372 mg TOS/kg bw/day in males; and 138, 292, and 447 mg TOS/kg bw/day in females. No treatment-related effects were observed on feed/water consumption, body weights, haematology, clinical chemistry, oestrous cycles, ophthalmology, or functional observations (functional performance or sensory reactivity). No treatment-related macroscopic or histopathological findings were observed at necropsy.

It was concluded that dietary administration of transglutaminase was well tolerated with no evidence of any adverse findings at any of the administered concentrations. The no observed adverse effect level (NOAEL) was therefore 5625 ppm (equal to 372 mg TOS/kg bw/day in males and 447 mg TOS/kg bw/day in females), the highest dose tested.

Genotoxicity

Bacterial Reverse Mutation Test (Treat-and-wash method (Covance Laboratories Ltd, 2020a). Regulatory Status: GLP; conducted according to OECD TG 471

The potential mutagenicity of transglutaminase (batch PPN66927, 7.5% TOS) was evaluated in *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535 and TA1537, and in the *Escherichia coli* strain WP2 *uvrA*, with and without metabolic activation using rat liver homogenate (S9 mix). Appropriate positive control articles, as recommended by the OECD guideline, were used. Water was used as the vehicle/negative control.

Two independent mutation tests were performed according to the treat-and-wash method: the plate incorporation method (experiment I) and the pre-incubation method (experiment II). All tests were conducted in triplicate. Concentrations of transglutaminase up to 5000 µg TOS/mL were tested. Other concentrations used were a series of *ca* half-$\log_{10}$ dilutions (diluted with water). No precipitation or toxicity was observed at any concentration of the test article.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed at any concentration level, with or without S9 mix, in both experiments I and II. The expected increases in revertant colony numbers were observed with all the positive control articles used, confirming the validity of the assay.

It was concluded that transglutaminase showed no evidence of mutagenic activity under the conditions of the assay.

In Vitro Micronucleus Test in Human Lymphocytes (Covance Laboratories Ltd, 2020b). Regulatory status: GLP; conducted according to OECD TG 487

The potential of transglutaminase (batch PPN66927, 7.5% TOS) to induce micronuclei formation in mammalian cells was tested using human lymphocytes isolated from peripheral blood, collected and pooled from two healthy, non-smoking adult volunteers (aged 18-35 years; of unspecified sex).

The study consisted of a preliminary toxicity study (to determine concentrations for the main test) and a main test, with test procedures being the same. In the main test, lymphocyte cultures were treated with transglutaminase either in the presence or absence of rat liver homogenate (S9 mix) for 3 hours; or treated for 20 hours in the absence of S9 mix. Before

treatment with transglutaminase, lymphocyte cultures were stimulated with phytohaemagglutinin. Following treatment (3-hour exposure) or during treatment (20-hour exposure), cytokinesis was blocked with cytochalasin B.

Transglutaminase concentrations up to 3000 µg TOS/mL were tested, dosed at 10% v/v. The vehicle/negative control was water; the positive controls were mitomycin C and colchicine in the absence of S9 mix, and cyclophosphamide in the presence of S9 mix. Cultures were performed in duplicate.

Transglutaminase did not cause any statistically significant increases in the frequency of micronucleated binucleate cells compared with vehicle controls, either in the presence and absence of S9 mix following 3-hour treatment; or in the absence of S9 mix following 20-hour treatment. All positive controls induced significant increases in the proportion of cells with micronuclei, confirming the validity of the test system.

It was concluded that transglutaminase did not induce micronuclei under the conditions of the study.

3.3.4 Potential for allergenicity

The applicant provided details of recent searches (2021) for amino acid sequence homology of the transglutaminase enzyme to known allergens, using the FARRP allergen protein database[8], using four sequence alignments: the full length protein (more than 35% identity), an 80 mer sliding window (more than 35% identity), a scaled 80 mer sliding window (more than 35% identity), and an 8 mer sliding window (100% identity).

No homology to sequences of known allergens was identified using these search parameters.

The applicant concluded that oral intake of transglutaminase is not anticipated to pose any food allergy concern.

3.3.5 Assessments by other regulatory agencies

There are no known safety assessments by other regulatory agencies.

The applicant provided documentation to show that the enzyme is approved by the Danish Veterinary and Food Administration. Although documentation is provided from the Danish Veterinary and Food Administration, this is just in the form of a certificate to Novozymes, certifying that the transglutaminase enzyme is:

- *suitable as ingredients to food production;*
- *fit for human consumption or for production of food intended for human consumption;*
- *and can be placed on the EU market without any restrictions.*

The documentation is not a safety assessment report.

3.4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a ‘worse-case scenario’ approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the transglutaminase

[8] AllergenOnline: http://www.allergenonline.org/

enzyme preparation remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al., 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an ADI or a NOAEL to estimate a margin of exposure for risk characterisation purposes. Whilst the budget method was originally developed for use in assessing food additives, it is also appropriate to use for estimating the TMDI for processing aids (FAO/WHO 2020). The method is used by international regulatory bodies and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO, 2021) for dietary exposure assessments for processing aids.

In their budget method calculation, the applicant made the following assumptions:

- the enzyme activity is expressed as transglutaminase units (TGHU(A)) per gram and 30 mg TOS is present in 200 TGHU(A) per gram enzyme preparation
- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed
- 1 kg flour produces 1.6 kg bread
- 1 kg pasta flour produces 3.8 kg pasta
- cheese contains 25% protein
- fermented dairy products and dairy analogues contain 10% protein
- egg substitute contains 12% protein
- meat and fish analogues contain 24% protein
- meat and fish products contain 30% protein
- among all solid foods, cheese produced the highest theoretical enzyme exposure when each solid food was assessed individually. Therefore the enzyme preparation use level and protein content of cheese was used in the budget method calculation to represent all processed solid foods
- the maximum physiological requirement for liquid is 100 mL/kg body weight/day (the standard level used in a budget method calculation for non-milk beverages)
- 25% of non-milk beverages are processed
- the densities of non-milk beverages are ~ 1
- among all non-milk beverages, use in brewing (beer) was the only use presented for beverages, therefore the enzyme preparation use level for beer was used in the budget method calculation for all processed non-milk beverages
- all of the TOS from the enzyme preparation remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 2.425 mg TOS/kg body weight/day.

As assumptions made by the applicant differ from those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI in two ways:

- Firstly, using the following assumptions that are highly conservative and reflective of a first tier in estimating dietary exposure:
  - The maximum physiological requirement for solid food (including milk) is 50 g/kg body weight/day (the standard level used in a budget method calculation where there is potential for the enzyme preparation to be in baby foods or general purpose foods that would be consumed by infants).
  - FSANZ would generally assume 12.5% of solid foods contain the enzyme preparation based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO, 2009). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst case scenario.
- Secondly, using the following assumptions that are also conservative, but more representative of actual food consumption patterns and are a second tier or refinement of the estimate of dietary exposure:
  - The maximum amount consumed of solid food (including milk) is 20 g/kg body weight/day (based on the Australian 2011-12 National Nutrition and Physical Activity Survey (NNPAS) consumption data).
  - FSANZ would generally assume 12.5% of solid foods contain the enzyme preparation based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO, 2009). However, the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion for solid foods as a worst case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI of the TOS from the enzyme preparation based on FSANZ's calculations was 4.8 mg TOS/kg body weight/day for the first tier calculation using consumption data based on physiological requirements. The TMDI for the second tier refined calculation based on actual consumption amounts was 2.0 mg TOS/kg bw/day.

The second tier refined TDMI is closer to actual dietary exposure over a long period of time, or over a lifetime given it is based on actual total food and beverage consumption amounts from nutrition survey data.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatisms in the budget method. This includes that it was assumed that all of the TOS from the enzyme preparation remains present and active in the final foods whereas the applicant has stated that the enzyme would be denatured or removed during processing and does not perform any technological function in the final food. In the processes where the enzyme is not denatured by heat or removed, the substrate is depleted or the conditions, such as pH, have changed which means that the enzyme will be inert.

4 Discussion and Conclusion

FSANZ concludes that the use of this transglutaminase as a processing aid for use in the nominated foods is consistent with its typical function of forming an isopeptide bond between γ-carboxamide groups of glutamine residue side chains and the ε-amino groups of lysine residue side chains. The technological purpose of transglutaminase as a processing aid has been provided for the nominated foods. FSANZ also concludes that the evidence presented to support its proposed use provides adequate assurance that the use of the enzyme, in the quantity and form proposed to be used (which must be consistent with GMP), is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

FSANZ has previously assessed the safety of *B. licheniformis* for a number of food processing aids (both GM and non-GM).

No public health or safety concerns were identified in the assessment of transglutaminase produced by GM *B. licheniformis* under the proposed use conditions. *B. licheniformis* has a long history of safe use as a source of enzyme processing aids, including several that are already permitted in the Code. The *B. licheniformis* host is neither pathogenic or toxigenic. Analysis of the modified production strain confirmed the presence and stability of the inserted DNA. Bioinformatics analysis indicated that the produced enzyme does not have substantial homology with known toxins or food allergens.

Toxicity testing of the transglutaminase enzyme showed no evidence of genotoxicity *in* vitro and the NOAEL in a 90-day oral toxicity study in rats was 372 mg TOS/kg bw/day, the highest dose tested. The theoretical maximum daily intakes (TMDI) calculated by FSANZ under two scenarios were 4.8 and 2.0 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDIs give MOEs of approximately 80 and 200, respectively. It was determined that the most appropriate estimate to use for the risk characterisation for this application, given it better reflects more realistic longer term dietary exposure, is the second tier calculation which gives an MOE of approximately 200.

Based on the reviewed data it is concluded that in the absence of any identifiable hazard an Acceptable Daily Intake (ADI) ‘not specified’ is appropriate.

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