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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting Document
Risk and technical assessment

A1292—Phospholipase C from GM *Bacillus licheniformis* as a processing aid

Executive summary

Food Standards Australia New Zealand (FSANZ) received an application from Novozymes Japan Ltd to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of phospholipase C (EC 3.1.4.3) from a genetically modified (GM) *Bacillus licheniformis* containing the phospholipase gene C from *Bacillus thuringiensis* as a processing aid in degumming vegetable fats and oils.

The proposed use of this phospholipase C as an enzyme processing aid in the quantity and form proposed is consistent with its typical function. Phospholipase C performs its technological purpose during food processing and does not perform its technological purpose in food for sale, therefore functioning as a processing aid for the purposes of the Code.

The enzyme preparation meets relevant identity and purity specifications.

No public health or safety concerns were identified concerning the use of the production organism, which is neither pathogenic nor toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

The enzyme preparation is not expected to pose a food allergenicity concern under the proposed conditions of use.

A no observed adverse effect level (NOAEL) of 714 mg total organic solids (TOS)/kg bw/day was identified in a 13-week oral toxicity study in rats. The theoretical maximum daily intake (TMDI) was calculated to be 0.1 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a Margin of Exposure (MOE) of approximately 7100.

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.

Overall, FSANZ concludes there are no safety concerns from the use of phospholipase C derived from GM *B. licheniformis* in the quantity and form proposed, consistent with its typical function in degumming of vegetable fats and oils.

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1. Introduction

Novozymes Japan Ltd has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of phospholipase C (EC 3.1.4.3) from a genetically modified (GM) *Bacillus licheniformis* containing the phospholipase C gene from *Bacillus thuringiensis* as a processing aid

The enzyme preparation is intended to be used as a processing aid in degumming vegetable fats and oils, at the minimum level required to achieve the desired effect, following Good Manufacturing Practice (GMP) principles.

The objectives of this risk and technical assessment were to:

- Determine whether the proposed purpose is solely technological and whether the enzyme preparation achieves its technological purpose as a processing aid in the quantity and form proposed.
- Evaluate potential public health and safety concerns that may arise from using this food enzyme preparation by considering the safety and history of use of the production organism and the safety of the enzyme.

Some information relevant to this assessment is Confidential Commercial Information (CCI) and cannot be disclosed.

2. Food technology assessment

2.1. Identity of the enzyme

The applicant provided relevant information regarding the identity of the enzyme, and this has been verified using the IUBMB[1] enzyme nomenclature reference database (McDonald et al. 2009). Details of the identity of the enzyme are provided below:

Accepted IUBMB name:	Phospholipase C
Systematic name:	Phosphatidylcholine cholinephosphohydrolase
Other names/common names:	Lipophosphodiesterase I, lecithinase C, *Clostridium welchii* α-toxin, *Clostridium oedematiens* β- and γ-toxins, lipophosphodiesterase C, phosphatidase C, heat-labile hemolysin, α-toxin
IUBMB enzyme nomenclature:	EC 3.1.4.3
CAS number:	9001-86-9
Reaction:	Phospholipase C catalyses the hydrolysis of phosphatidylcholine to diacylglycerol and phosphocholine. phosphatidylcholine + H_2O = 1,2-diacyl-*sn*-glycerol + phosphocholine

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 (Food and Agriculture Organization (FAO)/World Health Organization (WHO) 2020a).

The phospholipase C is produced by submerged, fed-batch fermentation of the genetically modified strain of *B. licheniformis*. Once fermentation is complete, the broth containing the enzyme undergoes primary separation and filtration to remove the solid biomass and residual cells of the production strain, then concentration to produce the final commercial enzyme preparation. The food enzyme is manufactured in accordance with GMP and the principles of Hazard Analysis and Critical Control Points (HACCP).

[1] International Union of Biochemistry and Molecular Biology.

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), referenced in subsection 3—2 of Schedule 3 of the Code. Enzymes used as a processing aid need to meet either of these specifications, or a relevant specification in section S3—3 of Schedule 3. In addition, under JECFA, enzyme preparations must meet the specifications criteria contained in the individual monographs. In the case of phospholipase C from *B. licheniformis*, there is no individual monograph[2].

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 the phospholipase C preparation. 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 applicant's liquid enzyme preparation (phospholipase C from B. licheniformis) compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes.*

Test parameters	*Test results[1]*	*JECFA[2]*	*Specifications Food Chemicals Codex[3]*	*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 25g)	Not detected	Absent	Negative	-
Escherichia coli (in 25g)	Not detected	Absent	-	-
Antimicrobial activity	Not detected	Absent	-	-
Production strain (cfu/g)	Not detected	Absent	-	-

[1] Where the results of all three batches are the same, only one is listed.
[2] Joint FAO/WHO Expert Committee on Food Additives (FAO JECFA Monographs 26 (2021).
[3] Food Chemicals Codex (2022).
cfu: colony forming units.

[2] For the functional use 'enzyme preparation', the JECFA database can be searched for individual monographs.

The specification for the enzyme preparation used by the manufacturer (as provided in section 2.5 of the application) includes a test for the absence of the production strain. The enzyme, however, is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. Refer to Section 3.4 below for the total organic solids (TOS) value. TOS encompasses the enzyme component and other organic material originating from the production organism and the manufacturing process, while excluding intentionally added formulation ingredients.

2.3. Technological purpose

Under the current application, phospholipase C from *B. licheniformis* is intended for use as a processing aid in edible oil products at a level up to 1000 PLC-S/kg[3] oils and fats. The applicant requested use of the enzyme at GMP levels.

As identified by the IUBMB (Section 2.1, above), phospholipase C catalyses the hydrolysis of phosphatidylcholine to diacylglycerol and phosphocholine. As explained in the application, in the production of edible oil products, the enzyme acts to remove major oil phospholipids (known as gums) to increase the total oil yield of the product. Phospholipase C converts phosphatides (phosphatidylcholine and phosphatidylethanolamine) to water-soluble esters (phosphorylcholine and phosphorylethanolamine, respectively), which are subsequently removed from the oil via centrifugation. Use of phospholipase C for the degumming of vegetable fats and oils enhances the stability of the product for storage or further processing and reduces the requirements for water in the manufacturing process.

For a schematic representation of the hydrolysis of phosphatidylcholine to diacylglycerol and phosphocholine catalysed by phospholipase C, refer to its record in the enzyme database BRENDA[4].

The technological purpose as stated by the applicant of phospholipase C from *B. licheniformis* in the manufacture of edible oil products is consistent with the typical function of phospholipase C and is supported by scientific literature, which indicates that this enzyme is principally for the degumming of vegetable fats and oils (Dayton and Galhardo 2008).

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 95°C. Therefore, the enzyme is inactivated in the processing of vegetable fats and oils and would have no technological effect in these foods after they are produced.

[3] PLC-S/kg: phospholipase C unit/kg

[4] EC explorer - BRENDA Enzyme Database (brenda-enzymes.org)

Table 2. Phospholipase C from B. licheniformis enzyme preparation physical and chemical properties.

Physical and chemical properties of commercial enzyme preparation	
Enzyme activity	>5000 PLC-S/g
Appearance	Liquid
Temperature range	Optimum activity within range 60–65°C[1]
Temperature stability	Maximum activity at 60°C after 30 minutes of incubation[1] Enzyme deactivated at 95°C after 30 minutes of incubation[1] Negligible activity remained at ≥80°C[1]
pH range and optimum	Activity within range pH 4–10[2,3] Optimum activity within range pH 8–9[2]

[1] Temperature range and stability determined at pH 7.
[2] pH range and stability determined at 30°C.
[3] Approximately 50% of activity retained between pH 6–10.
PLC-S/g: phospholipase C unit/g.

2.4. Allergen considerations

The applicant has advised that the raw material used during fermentation is consumed during fermentation, and any remaining amounts of the raw material will be removed in manufacturing processes, such as washing and filtration. The applicant has provided sufficient information to confirm that the raw material in the fermentation media is not present in the final commercial enzyme preparation. Additional constituents in the enzyme concentrate are not known food allergens.

2.5. Food technology conclusion

The use of this phospholipase C as a processing aid for use in the degumming of vegetable fats and oils in the manufacture of edible oil products is consistent with its typical function of catalysing the hydrolysis of phosphatidylcholine to diacylglycerol and phosphocholine. 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.

Phospholipase C performs its technological purpose during the production of the nominated foods, after which it is inactivated by heat, and is not performing a technological purpose in the final food. It is therefore functioning as a processing aid for the purposes of the Code.

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

This safety assessment aims to evaluate any potential public health and safety concerns that may arise from using this phospholipase C as a processing aid.

Some information relevant to this section is CCI, so full details cannot be provided in this public report.

3.1. Source microorganism

The host organism of the enzyme is *Bacillus licheniformis* Si3 lineage which was derived from a natural isolate of Ca63 strain, which has a long history of safe use by the applicant for production of food enzymes. Our assessment found the name *B. licheniformis* is validly published under the International Code of Nomenclature of Bacteria. *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). The European Food Safety Authority (EFSA) has granted *B. licheniformis* with qualified presumption of safety (QPS) status (EFSA BIOHAZ Panel et al. 2025). This microorganism also falls under 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 food and 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 several 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 *B. licheniformis* produced enzyme processing aids: serine proteinase (A1098), subtilisin (A1206), Alpha-amylase (A1219), and Beta-amylase (A1220), and transglutaminase (A1275).

Molecular data provided by the applicant confirmed the identity of the production strain *B. licheniformis*. 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 the production organism is not detected in the final enzyme production were provided by the applicant.

No public health and safety concerns were identified. The production strain is non-pathogenic, non-toxigenic, and does not contain any genetic material that could give rise to resistance to antibiotics.

3.2. Characterisation of the genetic modification

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

The gene that encodes the phospholipase C enzyme was chemically synthesised based on the sequence from *B. thuringiensis* available in public databases. Data provided by the applicant and analysed by FSANZ confirmed the identity of the phospholipase C enzyme.

The vectors used to transform the *Bacillus licheniformis* host strain are based on *Staphylococcus aureus* standard vectors. One vector contains the phospholipase C expression cassette consisting of a hybrid *Bacillus* promoter, the coding sequence for

phospholipase C and a hybrid *Bacillus* terminator. The second vector was used to remove a marker gene in the host strain. No elements of the vectors are left in the production strain.

The phospholipase C expression cassette was integrated at specific integration sites in the host strain. A transformant was screened for rapid growth and high phospholipase C activity, leading to the final production strain.

3.2.2. Characterisation of the inserted DNA

The final production strain does not contain any elements of the vectors, or any functional antibiotic resistance genes. The absence of genes of concern, and the presence of the inserted DNA, were confirmed by genome sequencing of the final production strain.

3.2.3. Genetic stability of the inserted gene

The applicant provided results of analyses of the relevant phenotypic characteristics (i.e. enzyme activity and protein synthesis) of the production strain in large-scale fermentation, to demonstrate the stability of the introduced DNA sequence. No instability of the strain was observed.

As all inserts are integrated into the genome and lack a functional origin of replication, they cannot be transferred by conjugation to other organisms, nor can fragments replicate autonomously. The inserted gene is therefore considered to be genetically stable.

3.3. Safety of the enzyme

3.3.1. History of safe use

There does not appear to be an established history of safe use for the specific phospholipase C that is the subject of this application. However, phospholipases as a functional group have a long history of safe use in food production. Several phospholipase A preparations have been approved for use as processing aids by FSANZ and included in Section 18 of the Code, as part of applications A501, A561, A1004, A1221 and A1246 (FSANZ 2004, FSANZ 2006, FSANZ 2009, FSANZ 2022a, FSANZ 2022b).

3.3.2. Bioinformatic assessment of homology with known toxins

The applicant performed a bioinformatics search to compare the similarity of the phospholipase C amino acid sequence to known toxins. The search was conducted using the National Center for Biotechnology Information (NCBI) Identical Protein Groups[5] database. No matches of concern were identified in the search.

3.3.3. Toxicology data

The applicant conducted several unpublished toxicological studies with their phospholipase C preparation which were reviewed in the present assessment:

- Bacterial reverse mutation assay
- *In vitro* mammalian cell micronucleus test
- 13-week oral toxicity study in rats.

[5] NCBI Identical Protein Groups

Genotoxicity studies

Two genotoxicity studies with the phospholipase C preparation were submitted. These studies were conducted in accordance with GLP and OECD Test Guidelines. The positive controls in these studies produced the expected responses. The results of these studies, as summarised in Table 3, showed no evidence of mutagenicity, clastogenicity or aneugenicity.

Table 3. *Genotoxicity studies of phospholipase C from B. licheniformis.*

Test[1]	*Test object*	*Concentration*	*Purity (% TOS)*	*Results*	*Reference*
Bacterial reverse mutation assay (OECD TG 471, [1997])	*Salmonella typhimurium strains* TA98, TA100, TA1535 and TA1537; and *Escherichia coli* strain WP2 uvrA pKM101	Experiment I[2]: 5, 16, 50, 160, 500, 1600 and 5000 µg TOS/plate Experiment II: 160, 300, 625, 1250, 2500, 5000 µg TOS/plate	10.5% w/w	Negative ± S9[5,6]	Ballantyne (2016)
Micronucleus tests in vitro (OECD TG 487, [2014])	Cultured human peripheral blood lymphocytes [1]	3+21 hour - S9[3]: 1000, 3000, 5000 µg TOS/mL 3+21 hour +S9[3]: 3000, 4000, 5000 µg TOS/mL 24+24 hour - S9[3]: 100, 500, 2000 µg TOS/mL[4]	10.5% w/w	Negative ± S9	Whitwell (2016)

[1] Human peripheral blood lymphocytes pooled from two male donors.
[2] Test conducted twice in triplicate.
[3] Test conducted in sets of duplicate cultures.
[4] Test conducted up to a concentration inducing 56% cytotoxicity.
[5] No observed increase in number of revertant cultures ≥2-fold (in strains TA98, TA100 and WP2 uvrA pKM101) or ≥3-fold (in strains TA1535 and TA1537) compared with the vehicle control.
[6] No historical control range available for *Salmonella typhimurium* strain TA1537 +S9.

Toxicity studies

13-week oral toxicity study in rats (Hughes 2016). Regulatory status: GLP; conducted in accordance with OECD Test Guideline (TG) 408 (1998)

Phospholipase C was administered to male and female Han Wistar Rats (RccHan; WIST strain) by oral gavage at doses of 109.8, 274.6 and 713.9 mg TOS/kg body weight (bw)/day, equivalent to doses of 10%, 25% and 65% of the test item, respectively. Deaths of two female rats, one in the low-dose (109.8 mg TOS/kg bw/day) group and one in the control group, were unrelated to the test article. There were no treatment-related clinical signs or adverse effects on any of the parameters evaluated. The no observed adverse effect level (NOAEL) in this study was 713.9 mg TOS/kg bw/day, the highest dose tested.

3.3.4. Potential for allergenicity

The applicant performed searches to compare the similarity of the phospholipase C amino acid sequence to known allergens. The searches were conducted using the Comprehensive Protein Allergen Resource (COMAPARE)[6] database. The search strategy included a search for >35% identity to known allergens in the phospholipase C sequence using stretches of 80 amino acids and over the full length of the alignment, which identified no matches to known allergens.

Based on the available information, the enzyme is not expected to pose a risk of food allergenicity.

3.3.5. Assessments by other regulatory agencies

The safety of phospholipase C from *B. licheniformis* has been evaluated by the EFSA (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) et al. 2020). The enzyme preparation has been approved for use as a processing aid in food in Denmark (2018) and Mexico (2019). The enzyme is also included in the positive list of approved processing aids in food for Brazil (2019) and France (2023).

The United States (US) Food and Drug Administration (FDA) has responded that it has 'no questions' to Novozymes' Generally Recognized as Safe (GRAS) notification of the phospholipase C produced by the GM *B. licheniformis* containing the phospholipase gene C from *B. thuringiensis* (FDA 2017). FSANZ notes that 'no questions' responses are not in themselves a safety assessment by the US FDA.

3.4. Dietary exposure

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worst-case scenario' approach to estimating likely levels of dietary exposure, assuming that all of the TOS from the 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 acceptable daily intake (ADI) or a NOAEL to estimate a margin of exposure (MOE) 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 2020b). The method is used by overseas regulatory bodies and the JECFA (FAO/WHO 2021) for dietary exposure assessments for processing aids.

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

- the maximum physiological requirement for solid food (including milk) is 25 g/kg body weight/day
- 50% of solid food is processed
- 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

[6] COMPARE

- all solid foods and non-milk beverages contain the highest use level of 11 mg TOS/kg in the raw material (vegetable fats and oils)
- all of the TOS from the enzyme preparation remains in the final food
- the final foods containing the specified amount of the TOS from the enzyme preparation would be consumed daily over the course of a lifetime.

Based on these assumptions, the applicant calculated the TMDI of the TOS from the enzyme preparation to be 0.0673 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 using the following assumptions that are 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 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 for solid food and non-milk beverages is 0.1 mg TOS/kg bw/day.

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 the TOS from the enzyme preparation remains in the final foods and beverages whereas the applicant has stated that it is likely to either be reduced or removed during processing or would be present in insignificant quantities. In addition, the enzyme would be inactivated and perform no function in the final food to which the ingredient is added.

3.5. Safety assessment conclusions

Sufficient information has been provided to assess the safety of the phospholipase C that is the subject of this application.

No public health or safety concerns were identified concerning the use of the production organism, which is neither pathogenic nor toxigenic. Analysis of the GM production strain confirmed the presence and stability of the inserted DNA.

No significant homology between the enzyme and any known toxins or allergens was identified. The enzyme preparation is not expected to pose a food allergenicity concern under the proposed conditions of use.

Phospholipase C showed no evidence of genotoxicity *in vitro*. There were no treatment-related clinical signs or adverse effects in a 13-week oral toxicity study in rats. The NOAEL in this study was 714 mg TOS/kg bw/day, the highest dose tested.

The TMDI was calculated by FSANZ to be 0.1 mg TOS/kg bw/day. A comparison of the NOAEL and the TMDI results in a MOE of approximately 7100.

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.

Overall, FSANZ concludes there are no safety concerns from the use of phospholipase C from *B. licheniformis* in the quantity and form consistent with its typical function in the degumming of vegetable fats and oils in the manufacture of edible oil products.

4. References

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