FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

Supporting Document 1

RISK ASSESSMENT REPORT

APPLICATION A1050 – ACYLTRANSFERASE FROM *BACILLUS LICHENIFORMIS* AS A PROCESSING AID (ENZYME)

Executive Summary

Background

The *Australia New Zealand Food Standards Code* (the Code) currently permits the use of numerous microbial enzymes as food processing aids. There is currently no permission for the use of acyltransferase as a food processing aid.

Application A1050 seeks approval for the use of glycerophospholipid cholesterol acyltransferase (designated KLM3′) derived from a genetically modified (GM) *Bacillus licheniformis*, as a processing aid.

The risk assessment has considered the technological suitability, the potential hazard and identity of the donor and host microorganisms, as well as assessing the potential hazard of the KLM3′ preparation. Based on the available data, no food safety concerns have been identified with the enzyme, or with the donor or host organisms used to produce the enzyme, which would preclude permitting its use as a food processing aid. The absence of any specific hazards being identified is consistent with KLM3′ undergoing normal proteolytic digestion in the gastrointestinal tract. The Application provides adequate assurance that the enzyme is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

The stated purpose for KLM3′ is for use as a processing aid to improve emulsification in:

- Egg yolk and whole eggs to avoid product separation during high temperature processing in the manufacture of mayonnaise.
- Processed meat products to improve the emulsification of fat in the product which improves consistency and reduces cooking loss.
- Degumming of vegetable oils.
- Production of UHT and powdered milk to reduce fouling.
- Yoghurt to facilitate fermentation and improve viscosity.
- Bakery products containing eggs to give a softer and more tender crumb.

The available data are considered sufficient to provide an acceptable level of confidence in the conclusions of this risk assessment in regard to the safety and suitability of the enzyme.

Conclusions

- *B. licheniformis* as the host organism is a well-characterised expression system for the production of enzymes, and has a long history of safe use.
- There was no evidence of KLM3′ toxicity at the highest dose tested in a 90-day repeat dose study. The No Observed Adverse Effect Level (NOAEL) was 41 mg/kg bw/day, the highest dose tested.
- There was also no evidence of genotoxicity.
- Based on the reviewed toxicological data it was concluded that in the absence of any identifiable hazard an ADI (Acceptable Daily Intake) 'not specified' is appropriate.
- Based on the available evidence, KLM3′ produced in *B. licheniformis* is considered safe for use in foods for human consumption.
- There is no negative impact on the lipid composition of foods produced using the enzyme.
- The stated purpose for this acyltransferase is to improve the emulsification properties of various foods. When used in the form and amounts prescribed, the enzyme is technologically justified and achieves its stated purpose.
- The enzyme meets international purity specifications.

Table of Contents

1. Introduction

An application, received from Axiome Pty Ltd on behalf of Danisco A/S, seeks approval to permit the use of a glycerophospholipid cholesterol acyltransferase enzyme (designated KLM3′) as a processing aid. The enzyme is produced from a genetically modified (GM) *Bacillus licheniformis* expressing a codon-optimised gene for a protein engineered variant of acyltransferase produced from *Aeromonas salmonicida* subsp. *salmonicida.* There are no current permissions for the enzyme in the Code.

The proposed use of KLM3′ is as a processing aid to improve emulsification in a range of foods and food manufacturing processes. The Applicant claims KLM3′ could replace or partially replace phospholipase and other emulsification agents currently used in:

- Egg yolk and whole eggs to modify phospholipids to lysophospholipids and cholesterol-esters in egg yolk which in turn avoids product separation at high temperature pasteurisation during production of mayonnaise.
- Processed meat products to improve emulsification which contributes to improved consistency and reduced cooking loss.
- Degumming of vegetable oils.
- Production of UHT and powdered milk to reduce fouling.
- Yoghurt to facilitate fermentation and improve viscosity.
- Bakery products containing eggs to give a softer and more tender crumb.

1.1 Objectives of the Assessment

In proposing to amend the Code to include an acyltransferase derived from a GM *B. licheniformis* as a processing aid, a pre-market assessment is required.

The objectives of this risk assessment are to determine:

- What are the potential public health and safety concerns that may arise from the use of KLM3′ as a processing aid?
- Is the proposed purpose clearly stated and does the enzyme achieve its technological function in the quantity and form to be added?

1.2 Risk Assessment Questions

The following risk assessment questions have been developed to address the objectives of the assessment:

- Does the enzyme preparation present any food safety issues?
- Does the enzyme achieve its stated technological purpose?

2. Characterisation of KLM3′

2.1 Identity of the enzyme

Systematic name:	phosphatidylcholine:sterol *O*-acyltransferase
IUMBM enzyme nomenclature:	EC 2.3.1.43
C.A.S. number:	9031-14-5
Other names:	phospholipid-cholesterol acyltransferase; LCAT (lecithin-cholesterol acyltransferase); lecithin:cholesterol acyltransferase; lysolecithin acyltransferase, acyltransferase BL1, glycerophospholipid cholesterol acyltransferase (GCAT), KLM 3′
Marketing name:	LysoMax® oil (for degumming of oils) and FoodPro ® Cleanline (for other applications).

2.2 Chemical and physical properties

KLM3′ enzyme is synthesised as a 317 amino acid protein, which in mature, secreted, processed form is 280 amino acids. The final enzyme preparation is a clear brown liquid with a pH of 5.7-6.8. The typical composition of the enzyme preparation is:

Enzyme solids (TOS)	8.67%
Ash	1.05%
Moisture	90.28%

2.2.1 Enzymatic properties

Acyltransferase is an enzyme that transfers acyl[1] groups from phospholipids and glycolipids to acceptors such as sterols (ie cholesterol and plant sterols), fatty alcohols and other smaller primary alcohols.

KLM3′, the acyltransferase enzyme described in this Application, primarily hydrolyses the following reaction:

Phosphatidylcholine + cholesterol → 1-acylglycerophosphocholine + a cholesterol ester

Depending on the food application, KLM3′ transfers the fatty acid moiety (ie palmitoyl, oleoyl or linoleoyl) from the *sn*-2 position in phosphatidylcholine to cholesterol or plant sterol.

In addition to the above reaction, acyltransferase also exhibits the enzymatic activities of phospholipase (EC 3.1.1.4) and lysophospholipase (EC 3.1.1.5). Phospholipase hydrolyses the ester bond in the *sn*-2 position of phosphatidylcholine to release a free fatty acid, while lysophospholipase performs the reverse reaction; esterification of a free fatty acid to the *sn*-2 position of lysophosphatidylcholine.

Enzyme activity, measured in terms of Lipid Acyl Transferase Units (LATU), is based on the enzyme's ability to hydrolyse lecithin and liberate free fatty acids. LATU is calculated as micromole fatty acid produced per minute under assay conditions by using an internal standard enzyme for calibration (Appendix A3 in the Application).

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[1] An organic radical having the general formula RCO, derived from the removal of a hydroxyl group from an organic acid

Under assay conditions (30°C, pH 7.0), fatty acids are cleaved from both the 1 and 2 positions and then measured via a commercially available kit containing a coupled enzyme scheme. The rate of fatty acid generation is proportional to the enzyme activity. The method is calibrated using a linear regression of standard dilutions prepared from a standard material that has been assayed by the Danisco reference method.
The KLM3′ enzyme has a minimum activity of 1000 LATU/g.

The Applicant has provided temperature, pH and stability curve analyses for the enzyme. Enzyme activity was determined after 10 minutes using lecithin as a substrate, at pH 7.0 over various temperatures. Temperature optimum was determined to be 65°C with activity rapidly declining at temperatures greater than 70°C. Less than 15% of optimal activity remained at 80°C.

Thermal stability was measured by determining residual activity after incubation for 30 minutes at 40-80°C under appropriate assay conditions. The enzyme is stable for 30 minutes at temperatures up to 60°C, while inactivation occurs at 70°C after 30 minutes incubation.

KLM3′ is active over the range pH 3 to 11 with optimum activity seen at pH 8.0. Optimal stability is seen between pH 8 to 10, and the enzyme is relatively stable in the pH range 5.0 to 10.0.

2.3 Production of KLM3′

The manufacturing process is a three-part process consisting of fermentation (growth of organism and production of enzyme), recovery (separation of cell mass from enzyme and concentration/purification of enzyme) and formulation (preparation of a stable enzyme formulation). The process follows standard industry practices and uses appropriate substrates and nutrients. Once fermentation is complete, the biomass is removed by centrifugation and/or filtration. The remaining fermentation broth containing the enzyme is then filtered and concentrated. Following concentration, the enzyme solution is standardised and stabilised with appropriate diluents and a final polish filtration step is applied. Full details of raw materials used are contained in Confidential Commercial Information (CCI) provided by the Applicant.

The production of KLM3′ is monitored and controlled by analytical and quality assurance procedures that ensure the finished product complies with the specifications and is of appropriate quality for use as a processing aid in food applications. KLM3′ is produced in accordance with good manufacturing practice (GMP). Residual amounts of gluten and soybean material, which are used as fermentation nutrients, may remain in the final enzyme preparation.

2.3.1 Description of the genetic modification

The production organism for the KLM3′ enzyme is *Bacillus licheniformis* strain GICC03265, which has been genetically modified to contain the gene for GCAT derived from *Aeromonas salmonicida* subsp*. Salmonicida*. The KLM3´ gene was modified at one amino acid position (asparaginase at position 80 has been changed to aspartic acid) and codon optimized for expression in *B. Licheniformis*. Full details of the gene, methods used in the genetic modification and recombinant microorganism are CCI. In brief, the modified KLM3´ gene was introduced into *B. licheniformis* on a vector derived from *Bacillus* plasmids pUBl10 and pE194. Integration into the *Bacillus* chromosome at the *cat* locus occurred by Campbell-type recombination. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences.

2.3.2 Identification of the donor and host (production) organisms

2.3.2.1 Donor organism - Aeromonas salmonicida subsp. Salmonicida.

The genus *Aeromonas* comprises Gram-negative bacteria that are wide-spread in aquatic environments. Some species of *Aeromonas* are known to be opportunistic pathogens, particularly for immunocompromised hosts, and have been associated with gastroenteritis, wound infections and septicaemia in humans. The donor organism of this application, *Aeromonas salmonicida* subsp. *salmonicida* is the causative agent of furunculosis, a bacterial septicaemia of salmonid fish. Due to its high host specificity to salmonids (Reith et al 2008), it has been classified by American Type Culture Collection (ATCC) as a biosafety level 1 organism (not known to cause disease in healthy adult humans).

2.3.2.2 Production organism - B. licheniformis

The safety of the production organism is an important consideration in the safety assessment for enzymes used as processing aids. The primary issue is the toxigenic potential of the production organism, that is, possible synthesis of toxins by the production strain, and the potential for the carryover of these into the enzyme preparation (Pariza & Johnson 2001).

The genus *Bacillus* consists of over 60 species of bacteria capable of producing endospores which are able of survive for extended periods under challenging environmental conditions. They are commonly found in the environment and as laboratory contaminants. Out of 3 major groups, two are Gram-variable, whereas one group is Gram positive (EPA 1997). Many *Bacillus* species are haemolytic. The diversity of this group is shown by the wide range of DNA base ratios of approximately 32 to 69 mol% G+C (Claus & Berkeley 1986), which is wider than usually considered for a genus (Norris et al 1981).

B. licheniformis is a mainly soil dwelling saprophyte[2]. It is thought to contribute to nutrient recycling due to the large range of enzymes produced. It is a facultative anaerobe classified as a member of the subtilis group or subtilis spectrum (Gordon 1973) comprising *B. subtilis, B. pumilus and B. licheniformis*. The three species are easily distinguishable using DNA homology information.

B. licheniformis Bra7 has been previously approved by FSANZ for production of the enzyme maltotetraohydrolase. Examination of ribotyping results and phylogenic trees suggest that this strain is closely related to reference strain type strain DSM13 (ATCC 14580).

2.4 Analysis and Specifications

As discussed in section 2.2.1, the activity of KLM3′, defined in LATU, is based on the enzyme's ability to hydrolyse lecithin and liberate free fatty acids. This method is used to assay KLM3′ in fermentation broths, concentrates and formulated products.

It is stated in the Application that the enzyme preparation is produced using appropriate GMP controls and processes to ensure the finished product does not contain any impurities of a hazardous or toxic nature.

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[2] A saprophyte is an organism that derives its energy by decomposing dead or decaying organic matter.

The Applicant has noted specifications for the commercial enzyme preparation (see Table 1) and provided three Certificates of Analysis which demonstrate conformance to the stated specifications (Appendix A5 in the Application).

Table 1: Specifications for the commercial enzyme preparation (as provided by the Applicant)

	Specification
Acyltransferase activity	900-1100 LATU/g
Appearance	Brown liquid
Lead	≤5 mg/kg
Total Viable Count	≤5 x 10^4 cfu/ml
Coliforms	≤30 cfu/g
Salmonella	Absent in 25 g
E. coli	Absent in 25 g
Antibiotic activity	Negative by test
Production Strain	Absent

Further, the Applicant states impurity and microbial specifications written for KLM3′ meet specifications laid down by the FAO/WHO Expert Committee on Food Additives (JECFA 2006). The monographs are primary reference sources listed in Clause 2 of Standard 1.3.4 – Identity and Purity.

Based on the provided information, FSANZ agrees that KLM3′ produced from a genetically modified strain of *B. licheniformis* meets international specifications for enzyme preparations.

3. Technological function

The KLM3′ enzyme's effectiveness is based on its effect on the cell membrane by transferring acyl groups from phospholipids and glycolipids to acceptors such as sterols (including cholesterol and plant sterols), fatty alcohols and other smaller primary alcohols. The acyl groups that will be transferred are mainly C14 to C18 fatty acids (myristic acid, palmitic acid, stearic acid, oleic acid, linoleic and linolenic acid). Cholesterol and other sterols accept the transferred acyl groups to become cholesterol-ester and sterol-esters. Which reaction products are formed depends on the available substrate, but generally consist of lysophospholipids, cholesterol-ester of C14 to C20 fatty acids and sterol-esters of C14 to C20 fatty acids (campesterol, stigmasterol, beta-sitosterol, 5-avenasterol and 7-stigmasterol).

3.1 Phospholipids

The term phospholipid may be used for any lipid containing phosphoric acid as a mono- or diester. Glycerophospholipid signifies any derivative of glycerophosphoric acid that contains an *O*-acyl, *O*-alkyl, or *O*-alkenyl group attached to the glycerol residue. All phosphoglycerols contain a polar head and two hydrocarbon tails and differ in size, shape and polarity of the alcohol component of their polar head. The fatty acids will also differ; usually one is saturated while the other unsaturated and location in the *sn*-2 position.

Common glycerophospholipids are named as derivatives of phosphatidic acid, such as 3-*sn*-phosphatidylcholine (common name, lecithin).

Polar lipids, such as lecithin, are important in food systems because of their ability to stabilise emulsions. Emulsions result when vigorous mixing of two immiscible liquids disperses droplets of one liquid throughout the other. Rapid breakdown occurs as the

dispersed droplets coalesce to form a layer which either floats to the surface or settles to the bottom – emulsion stability is enhanced by the presence of phospholipids (polar and non-polar regions).

3.2 Proposed use in food products

The proposed technological function of KLM3′ is to modify the phospholipids and glycolipids present in certain foods thereby improving emulsification in:

- egg yolk
- mayonnaise and cakes containing whole eggs
- degumming of oil
- processed meats
- UHT and powdered milk
- yoghurt.

3.2.1 Eggs

Egg yolk is a traditional emulsifying agent used in food.

The lipid content of whole eggs is almost exclusively present in the yolk which contains 32 to 36% lipid. Yolk lipids consist of about 66% triacylglycerols, 28% phospholipids and 5% cholesterol: the major phospholipids being phosphatidylcholine (73%) and phosphatidylethenolamine (15%) (Fennema 1996)

KLM3′ modifies phospholipid to lysophospholipid and cholesterol ester in egg yolk which, the Applicant claims, improves the yolk's emulsification properties. The Applicant also suggests the enzyme to be more economical for the producer; improves textural characteristics and improves the heat stability of the yolk, allowing for hot processing. When used in manufacture of sauces, dressing or mayonnaise, improved emulsification reportedly reduces product separation at high pasteurisation temperatures. Pilot scale trial data was submitted that demonstrated the egg content in mayonnaises can be reduced by 50% when using enzyme modified egg yolk and that use of this enzyme produces comparative results for oxidation stability to conventional phospholipase modified yolk.

To achieve the desired effects, the enzyme is proposed to be added to liquid egg for mayonnaise production at a maximum of 5000 LATU/kg and to liquid egg used in bakery products at a maximum of 500 LATU/kg.

3.2.2 Processed meat

The hydrolysis reaction of KLM3′ leads to the release of less hydrophobic and thus more water-soluble lysophospholipids, which have a higher dynamic surface activity in the aqueous phase. Lysophospholipids are excellent emulsifiers, and the oil-in-water emulsions stabilised by hydrolysed phospholipids show improved heat stability. Improved emulsification of the fat in meat can contribute to improvements in consistency and reductions in cooking loss.

The Applicant submitted studies examining the effect of the enzyme in a liver/oil emulsion, first in a model system and secondly in application trials of liver sausages with a high water and a high fat content respectively. Activity of the enzyme was also investigated by High Performance Thin Layer Chromatography (HPTLC) by measuring the presence of the phospholipids, phosphatidylcholine and phosphatidylethanolamine and lysophospholipids.

Improved emulsification was observed, indicated by lighter colour, in the enzyme treated liver/oil emulsions for both trials compared to the control. The raw liver trial also showed greater oil stability of the enzyme treated sample (0.07%) compared to the control (94% c.f. 51%).

In order to achieve the desired effects, a maximum addition amount of 300 LATU/kg is proposed for processed meat products.

3.2.3 Degumming oil

Crude vegetable oils like soybean oil contain 1-2% phospholipids that are removed by a degumming process during processing to improve oil quality and reduce sedimentation. Using KLM3′ to convert phospholipids to the more water-soluble lysophospholipids allows their removal by water washing, which, the Applicant claims is a more gentle process. During enzymatic degumming, free fatty acids are transferred to phytosterols forming phytosterol esters. Phytosterols are normally removed during oil refining processes. However, the Applicant notes they are not removed during enzymatic degumming processes due to the lower volatility of the sterol ester.

Enzymatic water degumming of oil changes the mass balance, giving increased oil yield compared to non-enzymatic processes. The Applicant claims the yield-enhancing ability of KLM3′ is the result of two interrelated features: the transferase reaction of the enzyme and the simultaneous formation of lysophospholipids, which improve the separation of the oil and gum phase.

The hydrolysis of fatty acids from phospholipids and subsequent binding to phytosterols reduces the amount of gum phase and increases the degummed oil phase. This increases oil yield as the phytosterol ester remains in the oil. Once the level of phytosterols in the oil is depleted, hydrolysis of phospholipids to lysophospholipids and free fatty acids improves the separation of phases and further contributes to increased oil yield. Formation of lysophospholipids also affects the consistency of the gum phase which is claimed to make separation of the oil and gum phase more efficient.

A maximum amount of 1000 LATU/kg has been proposed for use in degumming oil.

3.2.4 UHT and powdered milk

Fouling is a deleterious phenomenon which occurs during production of UHT milk and powdered milk. During thermal treatment, milk proteins are often denatured and precipitate to form fouling layers on the heat exchange surfaces. The Applicant claims use of this enzyme results in reduced fouling in the heat exchanger while still maintaining the high organoleptic quality of the enzyme treated milk.

Enzymatic treatment of milk with KLM3′ produces cholesterol-esters and lysolecithins. Production of lysophospholipids causes a reduction in the surface tension of the milk due to their participation in micelle formation. They also have the ability to interact with the milk protein (casein) in the micelle. Cholesterol which is normally oriented at the lipid membrane surface will now exist as cholesterol-esters in the bulk of the milk lipid particles. These effects (micelles containing lysolecithin and cholesterol-esters) are claimed by the Applicant to be responsible for the reduction in fouling during heat treatment of milk.

Fouling on the surfaces of plate heat exchanges can be detected through pressure drop. Results of the full scale factory data submitted clearly indicate that use of this enzyme in UHT milk extended the run time by several hours before a cleaning cycle was required.

In order to achieve the desired effects in UHT milk and milk products, a level of 5-20 LATU/kg is proposed.

3.2.4 Yoghurt

In yoghurt manufacture, production of lysolecithin and cholesterol-esters facilitates improvements in fermentation times through accelerated milk acidification. It is also claimed that the production of lysophospholipids trigger increased production of exopolysaccharides from lactic acid bacteria which increases viscosity.

Trial data submitted by the Applicant demonstrated improved fermentation times and effect on viscosity for milk treated with the enzyme compared to untreated milk.

3.3 Dietary context

The impacts of any changes to the lipid composition of the final food products as a result of the use of the enzyme were considered including whether such changes could have a negative effect on the blood lipid profile of consumers.

Of particular interest were any potential changes to the cholesterol content of the final food products as research indicates a positive relationship between dietary cholesterol intake and low density lipoprotein cholesterol concentration, resulting in an increased risk of coronary heart disease (Institute of Medicine & Food & Nutrition Board 2002). Data provided by the Applicant states that the efficiency by which the enzyme transfers fatty acids from phospholipids to cholesterol results in relative esterified cholesterol concentrations that are generally higher than found in most foods. Up to 95% of the free (unesterified) cholesterol is converted to cholesterol esters, depending on the food, processing conditions and enzyme dosage. In the general diet, naturally-occurring cholesterol esters comprise approximately 20% of total dietary cholesterol consumed (Rozner & Garti 2006). However, since cholesterol esters are hydrolysed by pancreatic esterase and absorbed in the intestine as free cholesterol (Shiratori & Goodman 1965; Rozner & Garti 2006), and the use of the enzyme will not increase the amount of total cholesterol in the final food product, the blood lipid profile of consumers of foods produced using this enzyme is not expected to be negatively affected.

This same enzyme reaction transfers fatty acids from phospholipids to phytosterols resulting in a significant relative increase in the amount of phytosterol esters in the final food product. Phytosterols occur in the diet in free, esterified, and glycosidic forms, and their absorption is less than 2% (Nissinen et al 2002; Rozner & Garti 2006). Phytosterols and their esters may be added to certain foods (including milk and yoghurt) (Standard 1.5.1) and there are no efficacy or safety issues related to their use at the permitted amounts. As ingested phytosterol esters are hydrolysed to free sterols and fatty acids in the upper intestine (Nissinen et al 2002), there are no concerns related to the relative increase in the amount of phytosterol esters in the final food product.

3.4 Conclusion

Data presented by the Applicant demonstrate the acyltransferase enzyme's effectiveness across a range of applications.

The stated purpose for this enzyme, namely for use as a processing aid to improve emulsification in a range of foods, is clearly articulated in the Application. The evidence presented to support the proposed uses, provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified and has been demonstrated to

be effective in achieving its stated purpose. Further, there will be no negative impact on the lipid composition of foods produced using this enzyme.

4. Hazard Assessment

The hazard of glycerophospholipid cholesterol acyltransferase (KLM3') was assessed by:

(1) considering the donor and host organisms, including any history of safe use in food production processes;

(2) examining the potential allergenicity of the encoded protein; and

(3) evaluating unpublished toxicity studies on the purified KLM3' protein.

4.1 Hazard of the donor and host organisms

4.1.1 A. salmonicida subsp salmonicida

As described in Section 2.3.2.1, the source organism of the introduced *gcat* gene is not known to be pathogenic to healthy humans as it possesses a high degree of host specificity (i.e. to fish). A secreted acyltransferase has been considered a key determinant of virulence of *A. salmonicida* subsp *salmonicida*, however, haemolysis has not been observed in intact mammalian cells. It has been demonstrated that while the individual glycerophospholipids found in human erythrocytes could serve as substrates for KLM3´, there was no difference in the haemolysis rate between human erythrocytes treated with KLM3´ and controls after 60 minutes. The enzyme did not penetrate the bilayers, but acted only on one side of the membrane (Buckley et al 1982). The preferred substrate for KLM3´ is phosphatidylcholine substituted with unsaturated fatty acids (Buckley et al 1982), with fish tissue having higher concentrations of polyunsaturated fatty acids than that of mammals (Lee & Ellis 1990).

4.1.2 B. licheniformis Bra7

Several strains *of B. licheniformis* have been implicated in human infection in immunocompromised individuals and neonates (EPA 1997). Toxin-producing isolates of *B. licheniformis* have been isolated from foods involved in food poisoning incidents, raw milk, and commercially-produced baby food. However, pathogenicity has been restricted to severely immunocompromised patients. Virulence is not generally associated with *B. licheniformis* and the similarity to *B. licheniformis* strain Bra7 to the reference strain DSM13 (ATCC 14580) further confirms non-pathogenicity of the host organism. All ATCC cultures of *B. licheniformis* are designated as bio-safety level 1 by ATCC (not known to cause disease in healthy adult humans).

B. licheniformis strain BRA7 is derived from a lineage that is well-characterised, non-toxigenic and non-pathogenic, and widely used to produce food-grade enzymes. FSANZ has previously assessed the safety of *B. licheniformis* as the source organism for a number of enzymes used as food processing aids. Standard 1.3.3 of the Code permits the use of the following enzymes derived from *B. licheniformis* or genetically-modified (GM) *B. licheniformis*: α-amylase, maltotetrahydrolase, pullulanase and serine proteinase.

Certificates of analysis submitted with the application indicated that *B. licheniformis* is not detectable in the final enzyme preparation to be used as a food processing aid.

4.2 Potential allergenicity of the encoded protein

Information provided in the Application on the use or KLM3' and subsequent food processing steps indicated that negligible levels of protein would be present in the final food. The Applicant has stated that any residual enzyme would be present as denatured protein and undergo normal proteolytic digestion in the gastrointestinal tract.

Bioinformatic analyses were undertaken by the Applicant on the degree of homology between the amino acid sequence of the KLM3′ protein and other proteins. A Basic Local Alignment Search Tool (BLAST) search was conducted on the mature KLM3′ amino acid sequence, which found significant homology ($p<0.01$) with over 300 protein sequences. These homologies were mostly shared with other acyltransferases and lipolytic proteins, as would be expected.

The FASTA algorithm was used to determine the degree of sequence alignment between the KLM3′ protein and known allergens contained in the Structural Database of Allergic Proteins (SDAP)[3] and Allermatch database[4]. The FASTA alignment threshold for potential allergenicity was 35% homology over 80 amino acids, which is consistent with the criterion established by the Codex Alimentarius (2003). This threshold aims to detect potential conformational IgE-epitopes. No significant homology with any known allergens was determined.

The potential allergenicity of the KLM3′ protein was further evaluated using a sliding window search for the presence of immunologically-relevant sequences of six contiguous and identical amino acids (i.e. linear IgE epitopes and possible T-cell epitopes). More frequently, stretches of 8 amino acids are used in this analysis in order to preclude false positives. Nevertheless, only one potential epitope was detected, which matched a peptide present in profilin allergens in timothy grass pollen, sunflower pollen and mugwort pollen. No matches were found with any known food allergens. To determine whether this epitope would be present on the surface of the KLM3′ protein, hydrophobicity analysis was undertaken using ExPaSy ProtScale software. This peptide was determined to be hydrophobic and therefore unlikely to be present on the outside of the folded protein and thus unlikely to be an antigenic epitope.

The conclusion from these bioinformatic analyses is that the KLM3′ protein does not show biologically relevant homology to any known allergen and on this basis is unlikely to be allergenic.

4.3 Evaluation of unpublished toxicity studies

Unpublished toxicity studies on the KLM3' protein were submitted by the Applicant and independently evaluated by FSANZ. These studies included acute and subchronic oral toxicity studies in rats, and *in vitro* and *in vivo* genotoxicity assays. The test material used in the acute and subchronic studies was the Ultra Filtrated (UF) concentrate (Lot No. 20068010) containing 1156 U/mL KLM3' and 30.40 mg total protein/mL [equivalent to 86.70 mg total organic solids (TOS)/mL], while lyophilized powder of the UF concentrate from Lot No. 20068010 was used in the genotoxicity studies. The test material was produced according to the procedure used for commercial production.

[3] http://fermi.utmb.edu/SDAP/
[4] http://www.allermatch.org/

4.3.1 Acute toxicity study

Proteins that cause toxicity act via acute mechanisms and generally at very low doses (Sjoblad et al 1992). Therefore, when a protein demonstrates no acute oral toxicity at a high dose level using a standard laboratory mammalian test species, this supports the determination that the protein will be non-toxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposures.

Jochumsen M (2006) Acute oral toxicity study in the rat: the fixed dose procedure. Study No. 62123. Lab: Scantox Lille Skensved, Denmark. Sponsor: Genencor International, Palo Alto, CA, USA. **GLP**: OECD. **QA Statement**: Yes. **Test Guidelines**: OECD Test Guideline 420.

In a range-finding experiment, KLM3' (Lot No. 20068010; 38 U/mg protein; 8.67% TOS; sourced from the Sponsor) was administered by oral gavage to one female HanTac:WH rat at 300 or 600 mg total protein/kg bw in 3% (w/v) aqueous sodium chloride vehicle. The dose volume was 20 mL/kg bw. In the main experiment, four female rats were dosed with 600 mg total protein/kg bw. Young rats (age unspecified) were sourced from Tactonic Europe (Denmark) and weighed 142-148 g at dosing. It was not specified in the study report whether rats were fasted prior to dosing. Food and water were available *ad libitum* following dosing. Clinical observations were made at 15 minutes, 1 hour, 3 hours and 6 hours following dosing and once per day for 14 days thereafter. Bodyweights were recorded prior to treatment on day 1, 2, 3, 8 and 15. Survivors were killed at the end of the observation period and necropsied.

There were no deaths. No clinical signs were observed in the range-finding study. At 3 hours after dosing, piloerection was observed in all rats in the main study, resolving by 6 hours. Two of the 5 rats dosed at 600 mg protein/kg bw lost a small amount of bodyweight (3-4 g) on day 2-3 but the overall bodyweight gain of all rats over 14 days was unremarkable. There were no treatment-related macroscopic abnormalities. The acute toxicity was therefore >600 mg total protein/kg bw (>1710 mg TOS/kg bw).

4.3.2 Subchronic toxicity study

Christensen A (2006) A 13-week oral (gavage) toxicity study in rats. Study No. 62129. Lab: Scantox Lille Skensved, Denmark. Sponsor: Genencor International, Palo Alto, CA, USA. **GLP**: OECD. **QA Statement**: Yes. **Test Guidelines**: OECD Test Guideline 408.

Experimental

KLM3' (Lot No. 20068010; 38 U/mg protein; 8.67% TOS; sourced from the Sponsor) was administered by oral gavage to 10 Sprague Dawley (Ntac:SD) rats/sex/group at 0, 4.56, 13.68 or 41 mg protein/kg bw/day for 13 weeks. The vehicle was 3% (w/v) sodium chloride and the dose volume was 5 mL/kg bw. Rats were sourced from Tactonic Europe (Denmark), were 5 weeks old and had a bodyweight range of $\pm$ 30 g for each sex at dosing. Food and water were available *ad libitum*.

Clinical signs were recorded daily, with a more detailed clinical evaluation performed weekly. Body weight and food consumption were recorded weekly, while water consumption was recorded twice weekly. Ophthalmoscopy was performed on all rats pre-treatment then in the control and high-dose groups during week 12. Blood samples were collected pre-treatment, on days 35 or 36, 65 or 66 and at termination for analysis of standard haematology and clinical chemistry parameters. Urine was collected pre-treatment and at termination for analysis of standard urinalysis parameters. At the end of the treatment period, survivors were killed and necropsied. Standard organs were weighed and histopathologically examined.

Findings

Five rats died or were sacrificed during the study at various times (0/20, 1/20, 3/20 and 1/20 at 0, 4.56, 13.68 and 41 mg protein/kg bw/day, respectively). The authors attributed the 3 deaths at the mid-dose to gavage error due to the presence of foreign material in the lungs. The remaining deaths were considered incidental in nature. There were no treatment-related clinical signs or effects on bodyweight gain. There was no treatment related effect on food or water consumption. Ophthalmoscopy was unremarkable. There was no treatment-related effect on any haematology, clinical chemistry or urinalysis parameter. There were no treatment-related macroscopic or microscopic findings. The No Observed Adverse Effect Level (NOAEL) was 41 mg total protein/kg bw/day (116.9 mg TOS/kg bw/day), the highest dose tested.

4.3.3 Genotoxicity

Two *in vitro* studies and one *in vivo* genotoxicity study were submitted as part of the current Application (Table 2). These studies were GLP compliant and conducted according to appropriate test guidelines. Signed QA statements were contained in the respective study reports. The two *in vitro* studies were conducted in the presence and absence of an exogenous source of metabolic activation (S9 liver preparations from Aroclor 1254-induced rats). Positive and negative (vehicle) controls were tested in each study and gave expected results. The enzyme preparation showed no evidence of mutagenic or clastogenic activity in these assays.

Table 2: Summary of genotoxicity studies

Test	Test system	Test article	Concentration or dose range	Result	Reference
Bacterial reverse mutation (Ames test)	*S. typhimurium* strains TA98, TA100, TA102, TA1535 & TA1537. (±S9)	KLM3' (Lot No. 20068010) Saline vehicle	0.16-5000 µg/plate	Negative Cytotoxicity at >500 µg/plate	Edwards (2006a)
Mammalian forward mutation	Human lymphocytes 3 or 20 h treatment (±S9)	KLM3' (Lot No. 20068010) Cell culture medium vehicle	0.0156-5000 µg/mL	Negative Cytotoxicity ≥128 µg/mL (-S9) & ≥0.5 µg/mL (+S9)	Edwards (2006b)
Mouse micronucleus	Mice (BomTAc:NMRI) PO, gavage 2 doses, 24 h apart. Killed 24 h after 2nd dose	KLM3' (Lot No. 20068010) Water vehicle	500, 1000 or 2000 mg/kg bw	Negative	Edwards (2006c)

S9 = 9000 × *g* supernatant from rat liver.

4.4 Conclusions

There are no public health and safety issues associated with the use of glycerophospholipid cholesterol acyltransferase (KLM3') as a food processing aid on the basis of the following considerations:

- The production organism is not toxigenic or pathogenic and is absent in the final enzyme preparation to be used as a food processing aid.
- Glycerophospholipid cholesterol acyltransferase would not be present in the final food.
- Bioinformatic analysis indicated that glycerophospholipid cholesterol acyltransferase is unlikely to be allergenic.
- Glycerophospholipid cholesterol acyltransferase was not acutely toxic in rats up to a dose of 600 mg/kg bw total protein (>1710 mg TOS/kg bw). The NOAEL following repeated oral dosing of rats was 41 mg total protein/kg bw/day (116.9 mg TOS/kg bw/day), the highest dose tested.
- Glycerophospholipid cholesterol acyltransferase was not genotoxic *in vitro* or *in vivo*.

Based on the absence of toxicity of the glycerophospholipid cholesterol acyltransferase an ADI 'not specified' is considered appropriate.

5. Dietary Exposure

Processing aids perform their technological function during the manufacture of food and are generally not present in the final food. Information contained in this application on the use of glycerophospholipid cholesterol acyltransferase and subsequent food processing steps, indicated that negligible levels would be present in the final food. Any traces of residual enzyme would be present as denatured protein and undergo normal proteolytic digestion in the gastrointestinal tract.

The Applicant has provided highly protective dietary exposure estimates based on United States and Danish consumption data. FSANZ accepts the submitted dietary exposure evidence, which supports a determination that further dietary exposure assessment is unnecessary.

6. Response to Risk Assessment Questions

Does the enzyme preparation present any food safety issues?

The Hazard Assessment reviewed evidence examining potential toxicity and genotoxicity associated with the enzyme preparation. There were no hazards identified which would preclude permitting use of the enzyme as a food processing aid.

No biologically significant homology was found between the KLM3′ protein and any known allergen. The final enzyme preparation is subject to labelling provisions of Standard 1.2.3 for the declaration of gluten and soybean as residual amounts of fermentation nutrients may remain in the commercial preparation.

The KLM3′ enzyme has not been evaluated by JECFA but has GRAS status in the United States (GRAS notice 265).

Does the enzyme achieve its stated technological purpose?

The stated purpose for this enzyme is as a processing aid to improve emulsification in a range of foods. The evidence presented to support this use provides adequate assurance that the enzyme, in the form and amounts added is technologically justified and has been demonstrated to be effective in achieving its stated purpose. Further, there will be no negative impact on the lipid composition of foods produced using this enzyme.

7. Conclusion

The risk assessment has considered the technological suitability, the potential hazard and identity of the donor and host microorganisms and the potential hazard of the glycerophospholipid cholesterol acyltransferase enzyme preparation.

The evidence presented was sufficient to determine that no safety concerns with the enzyme or host microorganisms exist. Thus glycerophospholipid cholesterol acyltransferase is unlikely to pose any health risk when used as a food processing aid. It was further concluded that the proposed use of the enzyme, namely as a processing aid to improve emulsification in a range of foods, was technologically justified in the form and prescribed amounts, and demonstrated to be effective. Further, there will be no negative impact on the lipid composition of foods produced using this enzyme.

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