Glycerolphospholipid Cholesterol Acyltransferase (KLM3´) Enzyme Preparation from a recombinant strain of *Bacillus licheniformis*

PROCESSING AID APPLICATION

Food Standards Australia New Zealand

Applicant: DANISCO AUSTRALIA PTY LTD
Submitted by: AXIOME PTY LTD

June 28, 2010

CONTENTS:

EXECUTIVE SUMMARY:

Danisco is seeking approval for a glycerophospholipid cholesterol acyltransferase (KLM3´) enzyme product for use in food products containing phospholipids including eggs and egg products, vegetable oils, processed meats, bakery products, and UHT milk and milk products. The enzyme is herein designated as KLM 3'.

KLM3' is derived from a selected non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* which is genetically modified to over express a glycerophospholipid cholesterol acyltransferase gene isolated from *Aeromonas salmonicida* subsp. *salmonicida*.

KLM3' will replace phospholipase and other emulsification agents currently used in food processing. KLM3' will be used in:

- Egg yolk and whole eggs to modify phospholipids to lyso-phospholipids and cholesterol-ester in egg yolk which in turn avoids product separation at high temperature pasteurization during production of mayonnaise;
- Processed meat products to improve the emulsification of processed meat product and contribute to better consistency and reduced cooking loss;
- Degumming of vegetable oils;
- Production of UHT and powdered milk to reduce fouling; and
- Bakery products containing eggs to give a softer and more tender crumb.

In all of these applications, KLM3' will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

To assess the safety of the *B. licheniformis* KLM3' for use in these applications, Genencor vigorously applied the criteria identified in the guidelines for food safety evaluations of enzyme preparations for use in food (Pariza and Johnson, 2001) utilizing enzyme safety data, the safe history of use of other enzyme preparations from *B. licheniformis*, the safe history of use of the production organism for the production of other enzymes used in food, and a comprehensive survey of the scientific literature.

In addition, different endpoints of toxicity were investigated at LAB Scantox (Denmark) and are evaluated and assessed in this document. KLM3' is non hazardous based on acute oral studies. In genotoxicity studies, KLM3' is not mutagenic, clastogenic or aneugenic. Daily oral administration of KLM3' up to and including a dose level of 41 mg total protein/kg bw/day or 116.90 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

Based on a worst-case scenario that a person is consuming KLM3' from the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, and in processed meat products (i.e., cumulative risk), this NOAEL still offers an 1051X fold margin of safety.

General information

1.1 Applicant details

(a) Applicant:

This application is made by Axiome Pty Ltd on behalf of Danisco A/S (Denmark)

(b) Company:

Danisco A/S

(c) Address:

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(f) Nature of Applicants Business:

Danisco A/S – manufacturer/marketer of specialty food ingredients, food additives and food processing aids.

Axiome Pty Ltd – regulatory & scientific affairs consultants

(g) Details of Other Individuals etc.:

No other individuals, companies or organizations are associated with this application.

1.2 Purpose of the application

This application seeks to modify Standard 1.3.3 Processing Aids to permit the use of a new *Processing Aid*, subject of this application.

This application is made solely on behalf of Danisco A/S, the manufacturer/marketer of the *Processing Aid*. When approved, the *Processing Aid* would be available for use by any Australian food manufacturer.

Approval of this application would require the following amendment to Standard 1.3.3 Processing Aids, Table to clause 17 – Permitted Enzymes of Microbial Origin:

Table 1: regulatory impact statement.

Acyltransferase EC 2. 3. 1. 43	*Bacillus licheniformis*, containing the gene for glycerophospholipid cholesterol acyltransferase isolated from *Aeromonas salmonicida* subsp. *salmonicida*

Currently no acyltransferase is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed in section 1.3.

KLM3´ derived from *B. licheniformis* carrying the gene encoding the glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *salmonicida* has been determined to be Generally Recognized as Safe (GRAS). It is also the subject of GRAS Notice 265 with a concurrence letter received from FDA, dated June 13, 2009. An application for use in food has been filed in Brazil and this is currently under review.

1.3 Justification for the application

KLM3´ is an enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding the glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida.* The enzyme is characterized as a phosphatidylcholine sterol O-acyltransferase (EC 2.3.1.43). A collection of information detailed in section 3 supports the safety of the production organism and the enzyme preparation for use in the applications outlined in section 4.

KLM3' will replace phospholipase and other emulsification agents currently used in food processing. KLM3' will be used in:

- Egg yolk and whole eggs to modify phospholipids to lyso-phospholipids and cholesterol-ester in egg yolk which in turn avoids product separation at high temperature pasteurization during production of mayonnaise;
- Processed meat products to improve the emulsification of processed meat product and contribute to better consistency and reduced cooking loss;
- Degumming of vegetable oils;
- Production of UHT and powdered milk to reduce fouling; and

- Bakery products containing eggs to give a softer and tenderer crumb.

More information on the benefit of this enzyme can be found in section 2.2.

Enzyme preparations are widely used as processing aids in the manufacture of food products. Currently no acyltransferase is permitted as a Processing Aid. Approval of this application would provide food processors with a new enzyme preparation offering the benefits and advantages as discussed previously.

1.4 Support for the application

No marketing or promotional activities have been undertaken for KLM3´ derived from *B. licheniformis* containing the gene for glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *salmonicida* in the Australian/New Zealand market. Hence at this stage, no requests from food manufacturers are provided in support of this application. However, the need and justification for use of the processing aid are discussed in section 1.3, and it is anticipated that support from the food processing industry will be submitted during the period for public comment on the application Draft Regulatory Measure/Assessment Report.

2 Technical information

Refer to Appendix A for further details

2.1 Type of processing aid

KLM3´ is an enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding the glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida.*

This Processing Aid falls into the category “Enzymes of microbial origin” from the Food Standard Code section 1.3.3.

2.2 Identity

a) Chemical/Common Name:

The systematic name of the principle enzyme activity is phosphatidylcholine sterol O-acyltransferase. Other names used are acyltransferase BL1, glycerophospholipid cholesterol acytransferase (GCAT), KLM3´.

- EC number: 2.3.1.43
- CAS number: 9031-14-5

Biological source: KLM3´ is an enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding the glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida.*

b) Marketing Name of the Processing Aid:

LysoMax® Oil (for degumming of oils) and FoodPro® Cleanline (for the other applications).

c) Molecular and Structural Formula:

KLM3´ is a protein. The amino acid sequence is known. Refer to Section 3.3.

2.3 Chemical and physical properties

KLM3´ is an enzyme that transfers acyl groups from phospholipids and glycolipids to acceptors such as sterols, fatty alcohols and other smaller primary alcohols. KLM3’ will be used as follows:

- To modify phospholipid to lysophospholipid and cholesterol ester in egg yolk, improving its emulsification properties and avoiding separation during pasteurization when the modified egg yolk is used in mayonnaise and bakery products;
- To convert meat phospholipids to lysophospholipids, improving consistency and reducing cooking loss by improved emulsification of the fat in the meat;

- To convert phospholipids to lysophospholipids which are more water-soluble and can be removed from the oil by washing with water during oil degumming to improve the quality of the oil and prevent sedimentation in the oil; and
- To transfer fatty acid acyl groups from milk phospholipid (or lecithin) to cholesterol resulting in the formation of lysophospholipids (or lysolecithins) and cholesterol esters, facilitating the formation of micelles and thereby reducing the build up of denatured proteins on heated surfaces during the production of UHT and powdered milk, and facilitating improved fermentation and viscosity formation during yogurt culturing.

Description: The commercial enzyme preparation is a brown liquid.

Substrate specificity:
KLM3´ catalyses the fatty acids transfer between phosphatidylcholine and sterols. Palmitoyl, oleoyl and linoleoyl residues can be transferred, and a number of sterols, including cholesterol, can act as acceptors.

Activity: The activity of the KLM3´ is defined in LATU (Lipid Acyl Transferase Unit). 1 LATU is defined according to an internal standard enzyme. The assay is based on the enzyme's ability to hydrolyze lecithin and liberate free fatty acids.

Temperature optimum: approximately 65°C.

Thermal stability: The enzyme is stable for 30 minutes at temperatures up to 60°C, while it is inactivated after 30 minutes of incubation at 70°C.

pH optimum: approximately pH 8

pH stability: Optimal stability is seen at the pH interval 8.0 to 10.0 and the enzyme is relatively stable in the pH range 5.0 to 10.0.

Interaction of the enzyme with different foods:
Refer above

Nutritional implication
KLM3´ is a protein and any residual amounts remaining in food consumed would accordingly have the same nutritional value. However, the use levels of KLM3´ are very low, and as with other enzymes that are currently approved and used as Processing Aids use of this product would not have any nutritional significance.

Particle size/distribution, morphology:
As KLM3´ is a liquid preparation, this information is not relevant.

2.4 Manufacturing process

The enzyme is produced by a submerged fermentation process using appropriate substrate and nutrients. When fermentation is complete, the biomass is removed by centrifugation/filtration. The remaining fermentation broth containing the enzyme is

filtered and concentrated. The concentrated enzyme solution is then standardised and stabilised with diluents. Finally, a polish filtration is applied.

Full details on the raw materials used for the production are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

The production of KLM3´ is monitored and controlled by analytical and quality assurance procedures that ensure that the finished product complies with the specifications and is of the appropriate quality for use as a processing aid in food processing applications.

2.5 Specification for identity and purity

Impurity profile:

Appropriate GMP controls and processes are used in the manufacture of KLM3´ to ensure that the finished product does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits are as follows:

Metals:	
Lead	less than 5 mg/kg
Microbiological:	
Total viable count	less than 5.10^{+4} CFU/g
Total coliforms	less than 30 CFU/g
E.coli	absent in 25g
Salmonella	absent in 25g
Antibiotic activity	negative by test
Production strain	absent
Physical properties:	
Appearance	liquid, brown

Standard for identity:
KLM3´ meets the specifications laid down by the Joint FAO/WHO Expert Committee on Food Additives and the Food Chemicals Codex.

Particle size/distribution, morphology:
As KLM3´ is a liquid preparation, this information is not relevant.

Allergenicity of the enzyme:
An allergen statement is given in Appendix A. Refer to Appendix B for additional information on the safety of the enzyme as to its allergenicity potential.

3 Safety

Refer to Appendix B for further details

3.1 Use of the enzyme as a food processing aid in other countries

KLM3´ derived from *B. licheniformis* carrying the gene encoding the glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *salmonicida* has been determined to be GRAS in the United States. It is also the subject of GRAS Notice 265 with a concurrence letter received from FDA, dated June 13, 2009.

Please refer to section 8.2 for details on the different approval procedures in the countries listed above.

3.2 Toxicity of the enzyme

To assess the safety of KLM3´, different endpoints of toxicity were investigated at Scantox laboratories (Denmark) and are evaluated and assessed in this document:

Study
Acute oral toxicity in rats – Fixed dose procedure
Sub-chronic 13 week toxicity in the rat
Ames mutagenicity study
In vitro chromosomal aberration Study
In vivo Mouse micronucleus study

The safety of KLM3´ has been assessed in toxicology studies investigating its acute oral, mutagenic and systemic toxicity potential. KLM3' is non hazardous based on acute oral studies. In genotoxicity studies, KLM3' is not mutagenic, clastogenic or aneugenic. Daily oral administration of KLM3' up to and including a dose level of 41 mg total protein/kg bw/day or 116.90 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects. A summary of the results of the studies can be found in Appendix B and the full test reports are attached in separate binders.

In addition, safety was further assessed according to the decision tree in the Pariza-Johnson guidelines (2001) for assuring the safety of a new enzyme preparation.

3.3 Information on the source micro-organism

KLM3´ is an enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding the glycerophospholipid cholesterol acyltransferase from *Aeromonas salmonicida* subsp. *Salmonicida.* The production organism of the KLM3´ enzyme preparation is *Bacillus licheniformis* strain GICC03265. It is derived by recombinant DNA methods from a strain of *Bacillus licheniformis* modified to express a synthetic DNA KLM3´ gene that has been modified at one amino acid and codon optimized for expression in *Bacillus licheniformis*. The host organism is *B. licheniformis* Bra7. *B. licheniformis* Bra7 was modified through deletion of several

enzyme activities (proteases, amylase), a sporulation gene and the native chloramphenicol resistance genes to make it suitable for expression of KLM3'.

Full details of the gene and recombinant microorganism are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

3.4 Pathogenicity and toxicity of the source micro-organism

KLM3' is derived from a selected non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* which is genetically modified to over express a glycerophospholipid cholesterol acyltransferase gene isolated from *Aeromonas salmonicida* subsp. *salmonicida*.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC, 1990). Pariza and Foster (1983) define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *B. licheniformis* meets these criteria for non-toxigenicity and non-pathogenicity.

The host organism, *B. licheniformis* Bra7, is a classical industrial strain used for enzyme production by Genencor, a Danisco division since 1989. The species *Bacillus licheniformis* is an accepted source of safe food enzymes in the literature. Its safety was recently reviewed (De Boer et al., 1994). The species *Bacillus licheniformis* is accepted as safe host for the construction of Risk Group I GMMs in several countries like Germany and The Netherlands, and exempted as a host under the NIH Guidelines in the USA. It has been classified as GRAS (Generally Recognized As Safe) by the U.S. Food And Drug Administration for the production of alpha-amylase per 21 CFR 184.1027, and other enzyme preparations.

The donor, *Aeromonas salmonicida* subsp. *salmonicida* is considered a Class 2 organism in the EU (see e.g. the DSMZ website) but is Biosafety level 1 in the USA (see ATCC website).

3.5 Genetic stability of the source organism

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by chloramphenicol resistance and KLM3´ production.

3.6 Method used in the genetic modification of the source organism

KLM3' is derived from a selected non-pathogenic, non-toxigenic strain of *Bacillus licheniformis* which is genetically modified to over express a synthetic

glycerophospholipid cholesterol acyltransferase gene originally isolated from *Aeromonas salmonicida* subsp. *salmonicida*.

The host organism is *B. licheniformis* Bra7. *B. licheniformis* Bra7 was modified through deletion of several enzyme activities (proteases, amylase), a sporulation gene and the native chloramphenicol resistance genes to make it suitable for expression of KLM3'

The donor organism is the bacterium *Aeromonas salmonicida* subsp. *Salmonicida.* The strain was obtained from the American Type Culture Collection as ATCC # 14174. The gene which encodes the glycerophospholipid-cholesterol acyltransferase (KLM3´) enzyme of the donor strain *Aeromonas salmonicida* subsp. *salmonicida* was synthesized according to the known DNA sequence, modified at one amino acid position, asparagine at position 80 has been changed to aspartic acid, and codon optimized for expression in *B. licheniformis*

Full details of the genetic modifications are provided in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4 Dietary exposure

Refer to Appendix C for further details

4.1 List of food or food groups likely to contain the enzyme or its metabolites

According to the food group classification system used in Standard 1.3.1-Food additives, KLM3´ will be used in:

- Dairy products excluding butter and fats (category 1 excluding category 1.6)
- Edible oils and fats (category 2)
- Bread and bakery products (category 7)
- Meat and meat products – processed meat (category 8.2)
- Egg and egg products (category 10).

4.2 Levels of residues in food

To obtain the desired effects, KLM3' will be added to eggs for mayonnaise production at a maximum of 5000 LATU/kg food; to processed meats at 300 LATU/kg food; to oil for degumming; to eggs used in bakery products at a maximum of 500 LATU/kg food and to UHT milk and milk dairy products at a level of 5-20 LATU/kg milk.

Estimated intake has been calculated for a range of foods at the maximum level using different sources:

- The Danish food survey 1995, Andersen, N.L., et al. 1996. Danskernes kostvarer 1995. Hovedresultater. Levnedsmiddel-styrelsen. Publikation nr. 235
- USDA, Results from USDA's 1994-96 Continuing Survey of Food Intakes by Individuals and 1994-96 Diet and Health Knowledge Survey
- Euromonitor international, national statistics, 2007
- European Food Safety Authority (EFSA), March 2008, Data Collection and Exposure Unit (DATEX), Belgian National Food Consumption Survey, reference year: 2004

Good agreement is demonstrated between the different data with maximum daily exposure of 0.039 mg total protein/kg body weight/day. The NOAEL has been determined for KLM3´ to be at 41 mg total protein/kg bw/day (equivalent to 116.90 mg TOS/kg bw/day). Based on a worst-case scenario of daily food consumption, the NOAEL would offer a 1051X fold margin of safety. It is important to mention that the total consumption of enzymes represents a "worst case" estimate. This estimate is also based on the scenario that (1) all above commodities are treated with KLM3', (2) 100% of KLM3' remains in the product after processing, and (3) all consumers eat all these commodities treated with KLM3'. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing.

4.3 Percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The product would be used as a processing aid in about:

- 5% of the tonnage of processed meat sold in Australia and New Zealand
- 20% of the tonnage of egg yolk contained in mayonnaise or bakery products such as cakes sold in Australia and New Zealand
- 4% of the tonnage of UHT milk and milk products sold in Australia and New Zealand
- 40% of the tonnage of degummed oils sold in Australia and New Zealand.

4.4 Levels of residues in food in other countries

Applications and levels of use of the KLM3´ preparation in other countries is the same as presented in section 4.2.

5 Assessment procedure

This application seeks to modify Standard 1.3.3 Processing Aids to permit the use of a Processing aid that is currently not permitted. Therefore, Danisco considers General Procedure Level 1 (up to 500 hours) to be the appropriate procedure for assessment of the application.

6 Confidential Commercial Information

Certain (identified) technical and manufacturing information included in Appendix E is regarded by the applicant as **Confidential Commercial Information** and is provided in the application strictly on this basis. This information is the result of a significant research and development effort and investment by the applicant; it is not in the public domain and is considered as either proprietary or commercially sensitive. It would be disadvantageous to the applicant if this information were released into the public domain.

7 Exclusive Capturable Commercial Benefit (ECCB)

There are patents pending in Australia and New Zealand in respect to specific food applications and the protein sequence. These patents are likely to provide ECCB for KLM3´ enzyme in ANZ. Note, however, that it is intended for payment for immediate evaluation of this application.

8 International and other National Standards

Refer to Appendix D for further details

8.1 Codex Standards

KLM3´ produced by *Bacillus licheniformis* has not been reviewed by JECFA; there is no specific Codex Standard relevant to this application.

8.2 International Legislation

KLM3´ has been determined to be GRAS in the United States as a food processing aid in degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products by a panel of scientific experts in the USA. In May 2009, the panel was reconvened to review the additional uses of the enzyme in UHT and powdered milk, and facilitating improved fermentation and viscosity formation during yoghurt culturing. It is also the subject of GRAS Notice 265 (which did not include the uses in UHT and powdered milk and yogurt) with a concurrence letter received from FDA, dated June 13, 2009.

GENENCOR
A Danisco Division

9 Statutory declaration

I, David Lloyd Bill,

of 4 Adelaide Street Balgowlah Heights, NSW 2094, regulatory affairs consultant:

make the following declaration under the *Statutory Declarations Act 1959:*

1) The information provided in this application fully sets out the matters required

2) The information provided in this application is true to the best of my knowledge and belief

3) No information has been withheld which might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Signature: _____

Declared at North Sydney on 28th of June 2010

Before me,

Signature: _____

JOYCE ELEANOR THORPE
Justice of the Peace Registration 138386
in and for the State of New South Wales, Australia

10 References

De Boer et al., On the industrial use of *Bacillus licheniformis* : a review. Appl. Microbiol. Biotechnol. 40 (1994) 595-598

IFBC (International Food Biotechnology Council). 1990. Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms in Biotechnologies and Food: Assuring the Safety of Foods Produced by Genetic Modification. Regulatory Toxicology and Pharmacology 12: S1-S196.

Pariza, M.W. and Foster, E. M. 1983. Determining the Safety of Enzymes Used in Food Processing. Journal of Food Protection, 46: 5: 453-468.

Pariza, M.W. and Johnson, E.A. 2001. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. Regulatory Toxicology and Pharmacology 33: 173-186.

Checklist

CHECKLIST FOR STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD

This checklist will assist you in determining if you have met the information requirements as detailed in the Application Handbook. Section 3.1 – General Requirements is mandatory for all applications. Sections 3.3.1-3.3.3 are related to the specifics of your application and the information required is in addition to section 3.1.

General Requirements (3.1)

- ☒ Form of application
- ☒ Applicant details
- ☒ Purpose of the application
- ☒ Justification for the application
- ☒ Information to support the application
- ☒ Assessment procedure
- ☒ Confidential Commercial Information
- ☒ Exclusive Capturable Commercial Benefit
- ☒ International standards
- ☒ Statutory Declaration

Food Additives (3.3.1)

- ☐ Support for the application
- ☐ Nature and technological function information
- ☐ Identification information
- ☐ Chemical and physical properties
- ☐ Impurity profile
- ☐ Manufacturing process
- ☐ Specifications
- ☐ Food labelling
- ☐ Analytical detection method
- ☐ Toxicokinetics and metabolism information
- ☐ Toxicity information
- ☐ Safety assessments from international agencies
- ☐ List of foods likely to contain the food additive
- ☐ Proposed levels in foods
- ☐ Percentage of food group to contain the food additive
- ☐ Use in other countries (if applicable)

Processing Aids (3.3.2)

- ☒ Support for the application
- ☒ Type of processing aid
- ☒ Identification information
- ☒ Chemical and physical properties
- ☒ Manufacturing process
- ☒ Specification information
- ☐ Industrial use information (chemical only)
- ☒ Information on enzyme use on other countries (enzyme only)
- ☒ Toxicity information of enzyme (enzyme only)
- ☒ Information on source organism (enzyme from micro-organism only)
- ☒ Pathogenicity and toxicity of source micro-organism (enzyme from micro-organism only)
- ☒ Genetic stability of source organism (enzyme from micro-organism only)
- ☒ Nature of genetic modification (PA from GM micro-organism only)
- ☒ List of foods likely to contain the processing aid

- ☐ Information on use in other countries (chemical only)
- ☐ Toxicokinetics and metabolism information (chemical only)
- ☐ Toxicity information (chemical only)
- ☐ Safety assessments from international agencies (chemical only)
- ☐ Anticipated residue levels in foods
- ☐ Percentage of food group to use processing aid
- ☐ Information on residues in foods in other countries (if available)

Nutritive Substances (3.3.3)

- ☐ Support for the application
- ☐ Identification information
- ☐ Information on chemical and physical properties
- ☐ Impurity profile information
- ☐ Manufacturing process information
- ☐ Specification information
- ☐ Analytical detection method
- ☐ Proposed food label
- ☐ Toxicokinetics and metabolism information
- ☐ Animal or human toxicity studies
- ☐ Safety assessments from international agencies
- ☐ List of food groups or foods likely to contain the nutritive substance
- ☐ Proposed maximum levels in food groups or foods
- ☐ Percentage of food group anticipated to contain nutritive substance
- ☐ Food consumption data for new foods
- ☐ Information on use in other countries
- ☐ Food consumption data for foods with changed consumption patterns
- ☐ Nutritional purpose
- ☐ Need for nutritive substance in food
- ☐ Demonstrated potential deficit or health benefit
- ☐ Consumer awareness and understanding
- ☐ Actual or potential behaviour of consumers
- ☐ Demonstration of no adverse affects to any population groups
- ☐ Impact on food industry
- ☐ Impact on trade

June 28, 2010

APPENDIX A: Technical information

Contents:

Appendices A

A1	IUB number
A2	CAS number
A3	Activity of the enzyme complex
A4	Specifications of the commercial product
A5	Certificate of analyses
A6	Production process flowsheet
A7	Food Chemical Codex, sixth edition
A8	FAO Food and Nutrition Paper - Monograph 3 (2006) (JECFA 67th Session) "General Specification for Enzyme Preparations used in Food Processing
A9	Allergen declaration

1 Identity

1.1 Acyltransferase

The systematic name of the principle enzyme activity is phosphatidylcholine sterol O-acyltransferase. Other names used are acyltransferase BL1, glycerophospholipid cholesterol acytransferase (GCAT), KLM3´,

The KLM3´ enzyme preparation is produced by submerged fermentation of *Bacillus licheniformis* expressing the KLM3´ gene from *Aeromonas salmonicida* subsp. *salmonicida*.

- EC number: 2.3.1.43 (Appendix A1)
- CAS number: 9031-14-5 (Appendix A2)

1.2 Other enzymes

Downstream processing concentrates and purifies the enzyme product. The resulting enzyme preparation will not be totally pure and trace of other enzyme activities (e.g. protease) might be found but their level will be very low.

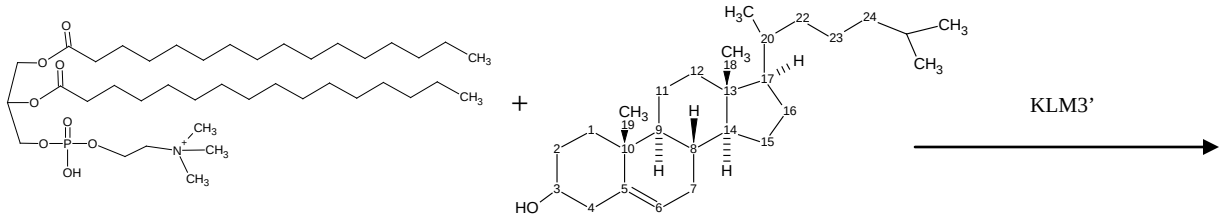
2 Chemical and physical properties

2.1 Substrate specificity

The enzyme catalyses the following reaction:

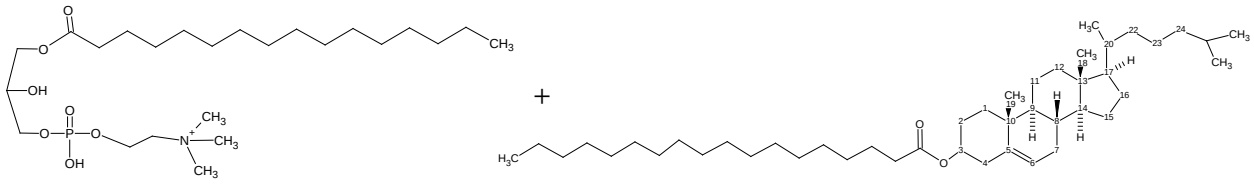
Phophatidylcholine + a sterol = 1-acylglycerophosphocholine + a sterol ester

KLM3' transfers the fatty acid moiety from the sn-2 position in phosphatidyl-choline to cholesterol.



Phosphatidylcholine

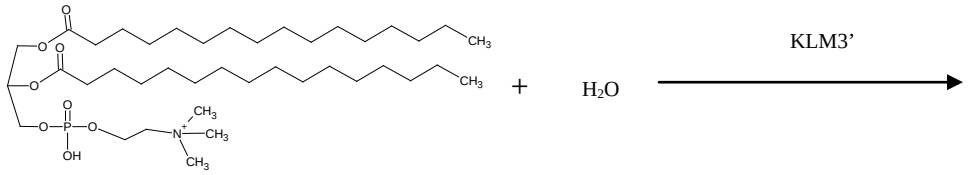
Cholesterol



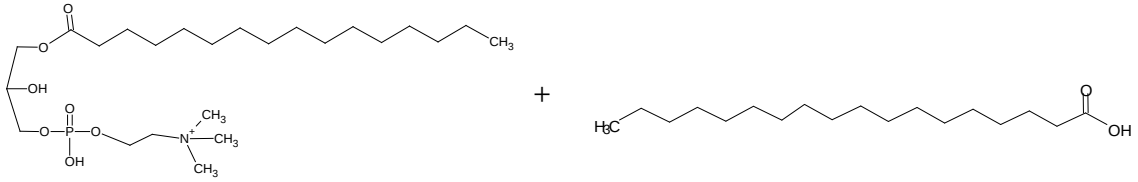
2-Lyso-phosphatidylcholine

Cholesterolester

KLM3' also hydrolyses the ester bond in the sn-2 position in phosphatidylcholine.



Phosphatidylcholine



2-Lyso-phosphatidylcholine

Free fatty Acid

2.2 Activity

The activity of the KLM3´ is defined in LATU (Lipid Acyl Transferase Unit). 1 LATU is defined according to an internal standard enzyme. The assay is based on the enzyme's ability to hydrolyze lecithin and liberate free fatty acids.

The assay is based on a 10-minute reaction of the enzyme with phosphatidyl choline. At 30°C and a pH of 7.0, phospholipase cleaves the fatty acids in both the 1 and 2 positions. The free fatty acids are then measured via a commercially available kit that contains a coupled enzyme scheme. The rate of fatty acid generation is proportional to the phopholipase activity. The method is calibrated using a linear regression of standard dilutions prepared from a standard material that has been assayed by the reference method (E) at Danisco. This method is utilized for assaying KLM3' (phospholipase) in fermentation broths, concentrates and formulated products.

KLM3´ has a minimum activity of 1000 LATU/g. A detailed assay method is present in Appendix A3.

2.3 Temperature optimum

Enzyme activity of purified KLM3' ferment was determined at various temperatures. The substrate was an emulsion of 0.6% phosphatidylcholine, 0.4% Triton X-100, 6 mM $CaCl_2$, and 50 mM HEPES, pH 7.0. Purified KLM3' ferment was diluted with 50 mM HEPES Buffer, pH 7.0 to 1.7 LATU/mL. 400 µl of substrate was thermostatted for 5 minutes at 30, 40, 50, 55, 60, 65, 70 ,80 and

90°C and 50 µl sample was added. After exactly 10 minutes enzymation was stopped by incubation at 99°C for another 10 minutes. Finally, the amount of free fatty acids was determined by the NEFA C method (Wako Chemicals GmbH, Neuss, Germany). Colour reagent A and B was made according to manufacturers protocol.10 µL substrate/enzyme mixture and 100µL reagent A were pipeppted to a microtiter plate and incubated at 37°C for 10 minutes. 200µL reagent B was added to the microtiter plate and incubated at 37°C for 10 minutes. The optical density at 540 nm was measured. The amount of free fatty acid was determined, using the read absorbance and a standard curve based on oleic acid. Results are shown in Figure 1.

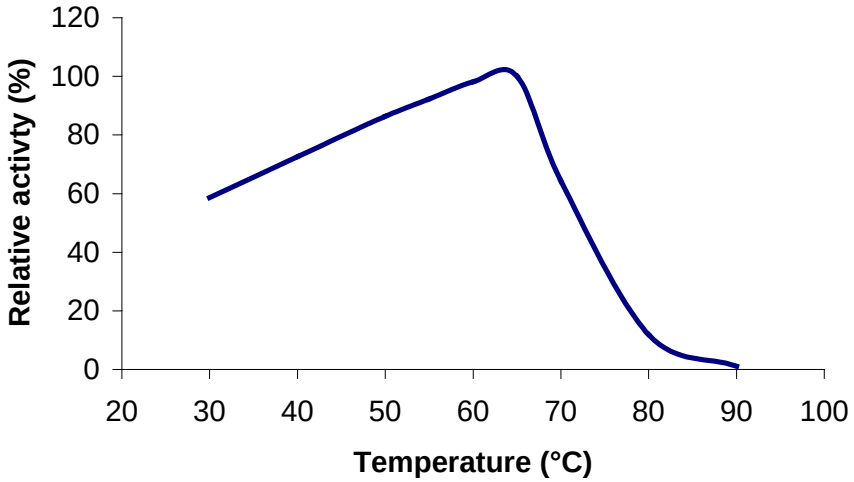


Figure 1: Activity was determined by 10 minutes of enzymation on lecithin substrate, pH 7.0, at various temperatures and subsequent determination of free fatty acids by the NEFA C method.

Temperature optimum was determined to be 65°C. At temperatures above 70°C the activity readily decreases and at 80°C less than 15% of the optimum activity is retained.

2.4 Thermal stability

Thermal stability of KLM3' was measured by determining residual lipase activity after incubation for 30 minutes at 40-80°C in 50 mM HEPES buffer, pH 7.0

Purified KLM3' ferment was diluted to 2.5 LATU/ml in 50 mM HEPES buffer, pH 7.0 and incubated for 30 minutes at 40, 50, 60, 70 and 80°C. The samples were stored on ice and residual activity was then determined by the following method: The substrate was an emulsion of 0.6% phosphatidylcholine, 0.4% Triton X-100, 6 mM $CaCl_2$ in 50 mM HEPES buffer, pH 7.0. 400 µl substrate was thermostatted for 5 minutes and 50 µl sample was added. After exactly 10 minutes enzymation

was stopped by incubation at 99°C for another 10 minutes. Free fatty acid in the substrate/enzyme mixture was analysed by using the NEFA C kit (999-75406, WAKO, Germany). Colour reagent A and B was made according to manufactures protocol. 10 µL substrate/enzyme mixture and 100µL reagent A were pipeppted to a microtiter plate and incubated at 37°C for 10 minutes. 200µL reagent B was added to the microtiter plate and incubated at 37°C for 10 minutes. The optical density at 540 nm was measured. The amount of free fatty acid was determined, using the read absorbance and a standard curve based on oleic acid. Results are shown in Figure 2.

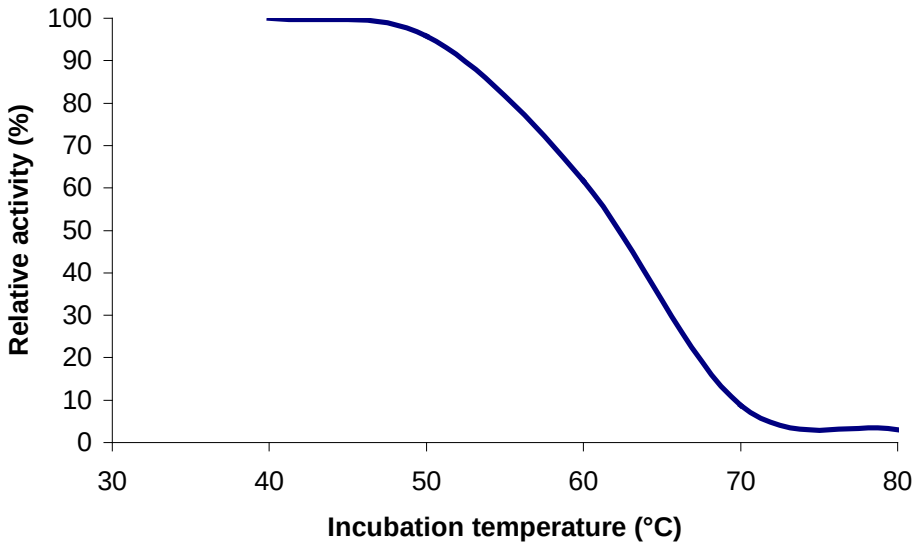


Figure 2: Activity was determined after 30 minutes of incubation in 50 mM HEPES buffer, pH 7 at 2.9 LATU/ml and various temperatures by subsequent 10 minutes enzymation on lecithin substrate at 37°C and pH 7.0 and finally determination of free fatty acids by the NEFA C method.

The enzyme is stable for 30 minutes at temperatures up to 60°C, while it is inactivated after 30 minutes of incubation at 70°C.

2.5 pH optimum

Enzyme activity of spray purified KLM3' ferment was determined at various pH-values. The substrate was an emulsion of 0.6% phosphatidylcholine, 0.4% Triton X-100, and 50 mM MacIlvaine buffer pH 4.0, 5.0, 6.0, 7.0, 7.6 and 8.0 as well as with 50 mM Tris buffer pH 7.6, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5 and 12.0. Compared to the determination of temperature optimum $CaCl_2$ was left out to prevent precipitation of calcium phosphate. This does not affect the enzyme activity. Spray purified KLM3' ferment was diluted with 50 mM HEPES Buffer, pH 7.0 1.7 LATU/mL. 400 µl of substrate was thermostatted at 37°C for 5 minutes and 50 µl sample was added. After exactly 10 minutes enzymation was stopped by incubation at 99°C for another 10 minutes. Finally, the amount of free fatty acids was determined by the NEFA C method (Wako Chemicals GmbH, Neuss,

Germany). Colour reagent A and B was made according to manufacturers protocol. 10 µL substrate/enzyme mixture and 100µL reagent A were pipeppted to a microtiter plate and incubated at 37°C for 10 minutes. 200µL reagent B was added to the microtiter plate and incubated at 37°C for 10 minutes. The optical density at 540 nm was measured. The amount of free fatty acid was determined, using the read absorbance and a standard curve based on oleic acid. Results are shown in Figure 3.

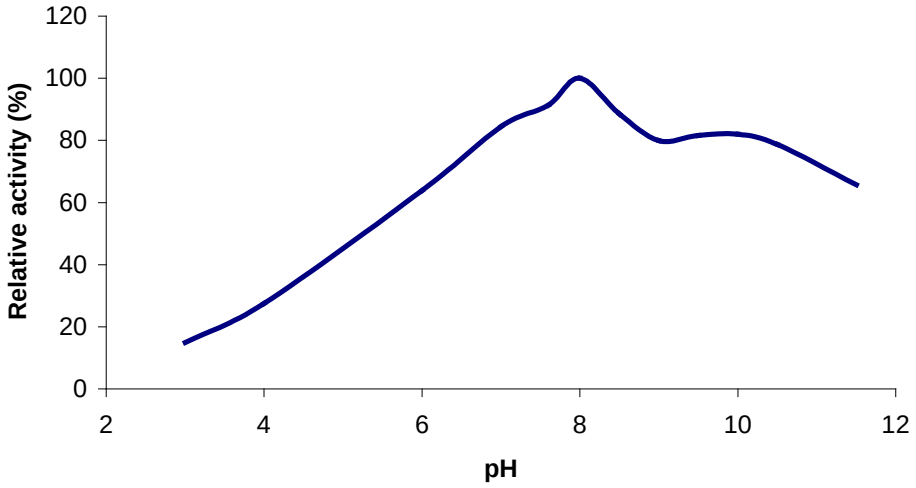


Figure 3: Activity was determined by 10 minutes of enzymation on lecithin substrate (without CaCl2) at 37°C and various pH (50 mM phosphate buffer) and subsequent determination of free fatty acids by the NEFA C method.

pH optimum is seen at pH=8.0 and the enzyme is active in the range from pH 3 to 11.

2.6 pH stability

Enzyme stability of purified KLM3' ferment was determined at various pH-values. Purified KLM3' ferment was diluted with 50 mM MacIlvaine buffer pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 as well as with 50 mM Tris buffer pH 8.0, 9.0, 10.0, 11.0, to 2.5 LATU/ml. After 30 minutes of incubation at 37°C the sample was stored on ice. Subsequently, residual phospholipase activity was determined by the following method: The substrate was an emulsion of 0.6% phosphatidylcholine, 0.4% Triton X-100, and 50 mM phosphate, pH 7.0. Compared to the determination of temperature stability $CaCl_2$ was left out to prevent precipitation of calcium phosphate. This does not affect the enzyme activity. 400 µl substrate was thermostatted for 5 minutes and 50 µl sample was added. After exactly 10 minutes enzymation was stopped by incubation at 99°C for another 10 minutes. Free fatty acid in the substrate/enzyme mixture was analysed by using the NEFA C kit (999-75406, WAKO, Germany). Colour reagent A and B was made according to manufactures protocol. 10 µL substrate/enzyme mixture and 100µL reagent A were pipeppted to a microtiter plate and incubated at 37°C for 10

minutes. 200µL reagent B was added to the microtiter plate and incubated at 37°C for 10 minutes. The optical density at 540 nm was measured. The amount of free fatty acid was determined, using the read absorbance and a standard curve based on oleic acid. Results are shown in Figure 4.

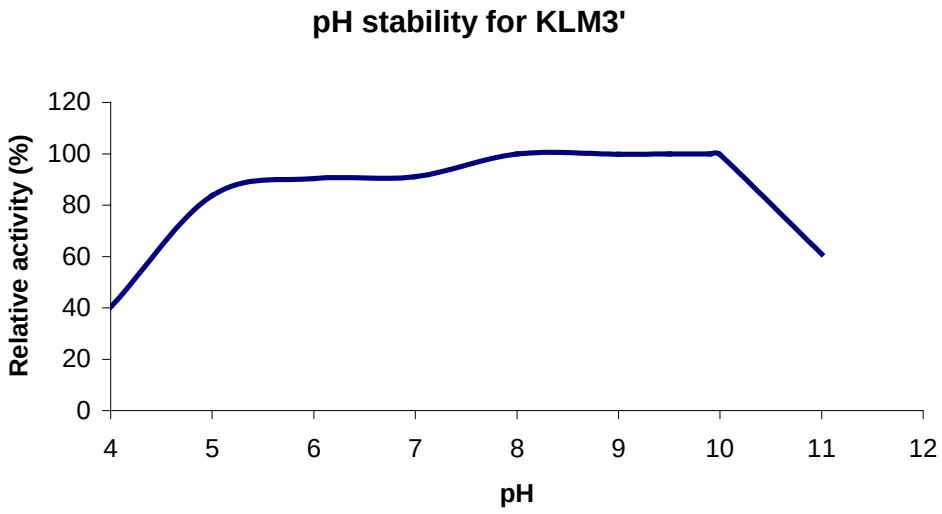


Figure 4: Activity was determined after 30 minutes of incubation in 50 mM MacIlvaine or 50 mM Tris buffer at 2.9 LATU/ml and various pH by 10 minutes of enzymation on lecithin substrate (without CaCl2) at 37°C and pH 7.0 and subsequent determination of free fatty acids by the NEFA method.

Optimal stability is seen at the pH interval 8.0 to 10.0 and the enzyme is relatively stable in the pH range 5.0 to 10.0.

2.7 Storage stability

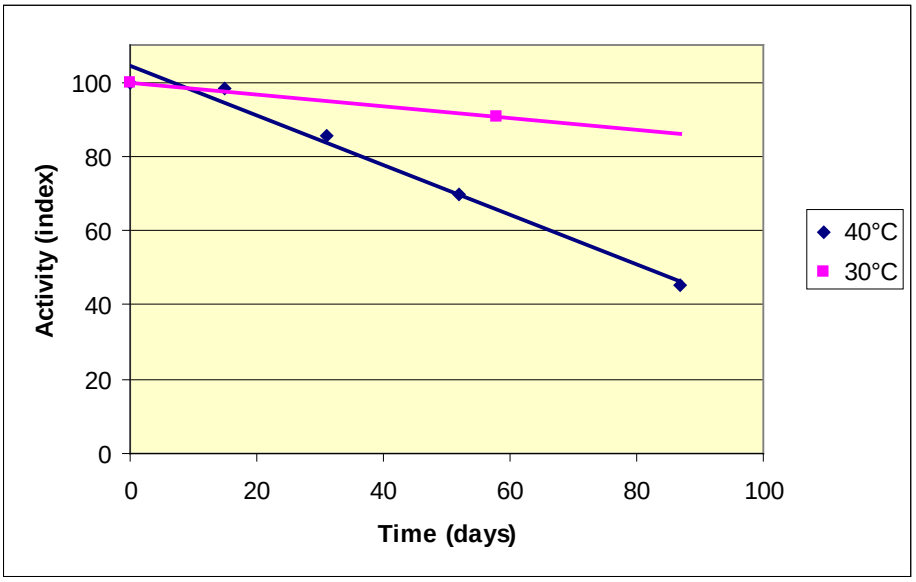


Figure 5: stability of the KLM3´ at 30°°C and 40C

At 5°C the enzyme is stable for more than 1 year without loss of activity.

3 Efficacy and benefits of the KLM3´ enzyme preparation

3.1 Description

KLM3´ is an enzyme that transfers acyl groups from phospholipids and glycolipids to acceptors such as sterols, fatty alcohols and other smaller primary alcohols. KLM3' will be used as follows:

- To modify phospholipid to lysophospholipid and cholesterol ester in egg yolk, improving its emulsification properties and avoiding separation during pasteurization when the modified egg yolk is used in mayonnaise and bakery products;
- To convert meat phospholipids to lysophospholipids, improving consistency and reducing cooking loss by improved emulsification of the fat in the meat;
- To convert phospholipids to lysophospholipids which are more water-soluble and can be removed from the oil by washing with water during oil degumming to improve the quality of the oil and prevent sedimentation in the oil; and
- To transfer fatty acid acyl groups from milk phospholipid (or lecithin) to cholesterol resulting in the formation of lysophospholipids (or lysolecithins) and cholesterol esters, facilitating the formation of micelles and thereby reducing the build up of denatured proteins on heated surfaces during the production of UHT and powdered milk, and facilitating improved fermentation and viscosity formation during yogurt culturing.

The KLM3´ will be used in:

- Degumming of oil
- Egg yolk
- Whole eggs for cakes
- Whole eggs for mayonnaise
- Processed meats
- UHT milk, powdered milk and yoghurt.

The effectiveness of this KLM3´ is based on its effects on the cell membrane by transferring acyl groups from phospholipids and glycolipids to acceptors such as sterols, fatty alcohols and other smaller primary alcohols. The acyl groups that will be transferred are mainly C14 to C18 from the fatty acids - myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. Cholesterol and other sterols accept the transferred acyl groups to become cholesterol-ester and sterol-esters. Fatty alcohols (defined as C12 and larger alcohols) can also be

esterified. The reaction products formed depend on the substrate(s), but generally consist of lyso-phospholipids, 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).

3.1.1 Egg yolk

Egg yolk is well known for use in the food industry due to its emulsification properties. Approximately 30% of the lipid in egg yolk is phospholipid, which contributes to egg yolks emulsification properties. In many foods including mayonnaise, sauces, dressings and cakes the emulsification properties of egg yolk are exploited. For some food applications, however, the emulsification properties of egg yolk are not sufficient to obtain a homogenous product without separation. In mayonnaise for instance pasteurization of the product at high temperatures causes the product to separate.

The enzyme preparation will be used to modify phospholipid to lyso-phospholipid and cholesterol-ester in egg yolk. Product separation at high temperature pasteurization can be avoided using enzyme modified egg yolk for production of mayonnaise.

3.1.2 Processed meat products

The enzyme preparation will be used in processed meat products. It will contribute to improve the emulsification of processed meat products and contribute to better consistency and reduced cooking loss.

The enzyme preparation added to processed meat will convert meat phospholipids to lysophospholipids. Because of the emulsification properties of lysophospholipids, this component contributes to improved consistency and cooking loss by improved emulsification of the fat in the meat. By the action of the enzyme in meat, lysolecithin is produced by the transfer reaction of fatty acids from phospholipids to cholesterol during formation of cholesterol esters. Cholesterol ester is not an unknown constituent of the diet as 8-15 % of the cholesterol in the diet is available as cholesterol esters.

3.1.3 Vegetable oil

Crude vegetable oils like soya bean oil contain 1-2% phospholipids, which are removed form the oil during the refining process, in order to improve the quality of the oil and prevent sedimentation in the oil. The removal of phospholipids is conducted by a so-called degumming process during the oil reefing process. The degumming can be conducted by chemical or enzymatic means. In the degumming process the enzyme will convert phospholipids to lysophospholipids which are more water-soluble and can be removed from the oil by washing with water. Enzymatic hydrolysis of phospholipids is a more gentle process compared

with the chemical degumming, which needs more acids and alkaline. The degumming with the enzyme will cause fewer effluents. During the degumming process the enzyme catalyzes the transfer of fatty acids form phospholipids to phytosterols in the oil during formation of phytosterol esters.

Phytosterols are normally removed by deodorization during the oil refining, but when the enzyme preparation is used the phytosterols are converted to phytosterol esters; these esters are not removed during the refining process because of lower volatility of the sterol esters. Phytosterol esters are not unknown constituents in vegetable oil because a smaller part of phytosterol exists naturally as esters.

3.1.4 Milk products

- Milk processing

During production of UHT milk, the heat exchanger is subject to a complex phenomenon which causes undesired material accumulation along its heating surfaces; this is normally referred to as fouling. Fouling of heated surfaces is also an issue during the manufacture of powdered milk. If the milk product is treated with KLM3', the enzyme will catalyze the transfer of fatty acid acyl groups from phospholipid (or lecithin) to cholesterol resulting in formation of lysophospholipids (or lysolecithins) and cholesterol ester. Production of lysophospholipids causes a reduction in the surface tension of the milk by their participation in the formation of micelles. Lysophospholipids also have the ability to interact with milk protein (casein) in micelles. Cholesterol which is normally oriented at the lipid membrane surface will be found as cholesterol esters in the bulk of the milk lipid particles. It is expected that the production of micelles containing lysolecithin and cholesterol ester causes the observed reduction in build up of denatured protein complexes at the heating surfaces of the heat exchangers during the production of UHT milk and the reduction in buildup of denatured protein on heating surfaces during heat treatment of powdered milk in heat exchangers and evaporators. It is also expected that the production of lysolecithin and cholesterol esters facilitates the observed improvement in fermentation by accelerating the acidification of the milk and production of yoghurt; furthermore it is expected that the production of lysophospholipids triggers an increased production of exopolysaccharide (EPS) from the Lactic Acid Bacteria giving rise to increased viscosity. The increased fermentation speed and increased viscosity in yoghurt produced from enzyme treated milk have been demonstrated in trials.

Heat treatment of the enzyme KLM3´during the normal UHT, powdered milk and yoghurt process causes a complete inactivation of the enzyme, and no activity is left in the final product.

3.1.5 Bakery products

In cakes, enzymatically modified egg yolk gives a softer and tenderer crumb.

3.2 Efficacy examples

3.2.1 In processed meat products

Acyltransferase KLM3´ reacts on the sn-2 ester bond of phosphatidylcholine, removing one fatty acid to the acceptor cholesterol. The reaction results in the formation of lysophospholipids and cholesterol ester. The hydrolysis leads to the release of less hydrophobic and thus more water-soluble lysophospholipids, which have a higher dynamic surface activity because of the higher unimer concentration in the aqueous phase. Lysophospholipids are excellent emulsifiers, and the oil-in-water emulsions stabilised by hydrolysed phospholipids show improved heat stability.

In Figure 6, it can be seen that KLM3´ highly improved the oil emulsion stability of the liver. At a concentration of 0.07% KLM3, the liver emulsion had an oil stability of 94% compared to 51% in the control. Also a much lighter colour is observed in the enzyme-treated liver/oil emulsion compared to the control, which demonstrated a better emulsion.

Figure 6: Pictures of raw liver emulsion
left: KLM3-treated liver/oil emulsion right: Control liver/oil emulsion

In the application trial (Figure 7), the KLM3-treated liver sausages with high fat have a lighter colour, harder texture and improved fat retention compared to the control and CITREM- treated liver sausage.

Figure 7: Pictures of heat-treated liver sausages

3.2.2 In degumming vegetable oil

The yield-enhancing ability of KLM3´ is the result of two interrelated features: the transferase reaction of the enzyme and the simultaneous formation of lyso-phospholipids, which improve the separation of the oil and gum phase. High quality degummed oils with phosphorus levels below market specifications are guaranteed.

Essentially, the transferase reaction controls the formation of free fatty acids, maintaining the high quality of the degummed oil. The binding of free fatty acids to phytosterols contributes further to increased oil yield, as the phytosterol ester remains in the oil. A secondary effect is that the enzyme hydrolyses phospholipids into lysophospholipids and free fatty acids, when the level of phytosterols in the oil is depleted. The conversion of phospholipids into hydratable lyso-phospholipids reduces the content of phosphorus in the oil, as the hydratable phospholipids are removed in the centrifugation step. Moreover, the formation of lysophospholipids has a favourable effect on the consistency of the gum phase, making the separation of the oil and gum phase more efficient. A further advantage of KLM3´ in oil processing is that it is not active on triglycerides.

Enzymatic water degumming of oil changes the mass balance, giving increased oil yield compared to a non-enzymatic process. This is due to the transferase reaction of the enzyme. The transfer of fatty acid from phospholipids to

phytosterols reduces the amount of gum phase and increases the degummed oil phase. Another factor contributing to increased oil yield is the transformation of phospholipids into lyso-phospholipids, which improves the separation of the oil and gum phase. Lyso-phospholipids are more water soluble than phospholipids and are, thus, more easily separated from the oil during centrifugation.

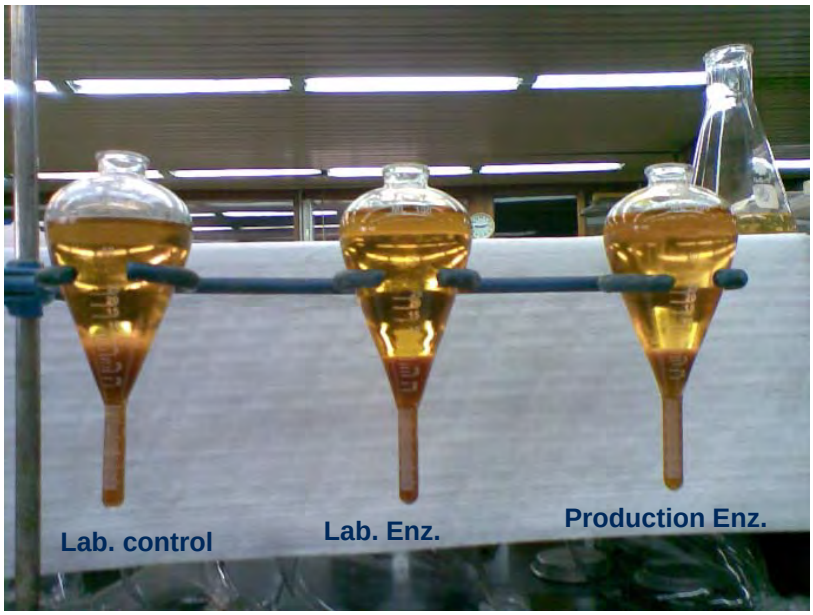


Figure 8: Water degumming of crude soya oil

3.2.3 In milk processing

During thermal treatments of milk or milk based products, proteins are often denatured and precipitated to form fouling layers on the heat exchange surfaces.

Essentially, the transferase reaction controls the formation of lysophospholipids without formation of significant amount of free fatty acids, because the amount of donor substrate phospholipid is lower than the amount of acceptor substrate cholesterol. This will secure the reduced fouling effect in the heat exchanger and still maintaining the high organoleptic quality of the enzyme treated milk.

Generally it is possible to detect the fouling of plate heat exchanger surfaces through a linear evolution of pressure drop and the overall heat transfer coefficient, and most commonly the UHT plants monitor development of pressure drop, in order to get an indication of when it is necessary to run an intermediate cleaning cycle (CIP).
In the Figure 9, development of pressure drop over a UHT plant is shown from actual full scale factory trials carried out over a longer period and average data collected.

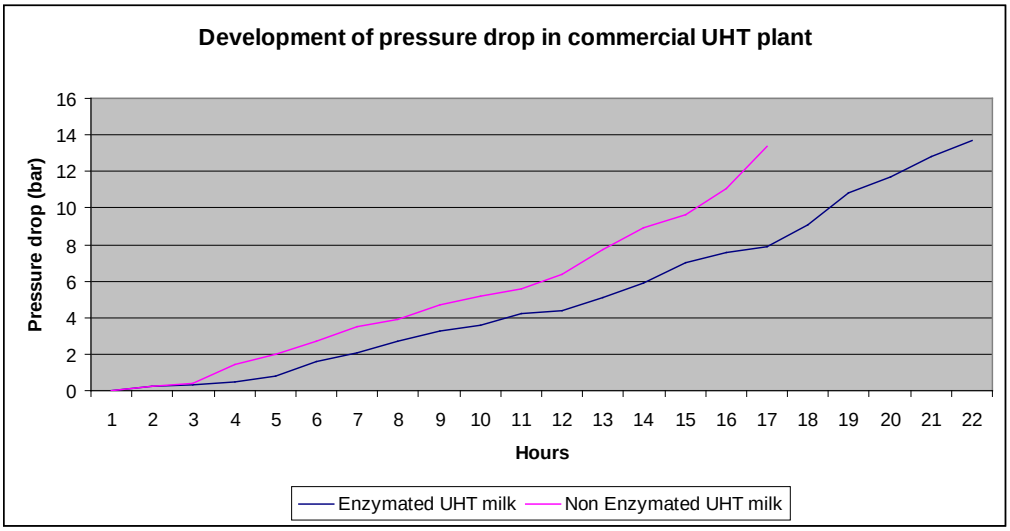


Figure 9: Development of pressure drop in UHT plant

From the full scale trial we have seen that the run time for a UHT plant can be increased with several hours, i.e. generating a large saving in cost of detergents, energy and manpower for CIP and at the same time leaving more actual production time on the processing plant.

4 Manufacturing process

The manufacturing process for the production of KLM3' will be conducted in a manner similar to other food and feed production processes. It is conducted in accordance with food good manufacturing practice (GMP) and the resultant product meets the general requirements for enzyme preparations of the Food Chemicals Codex, Sixth Edition (FCC 2008) and the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA, 2006).

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/ drying (preparation of a stable enzyme formulation). The production process follows standard industry practices (see, Enzyme Applications, 1994; Aunstrup et al, 1979; and Aunstrup, 1979).

4.1 Raw materials

The raw materials used in the fermentation and recovery process for the KLM3´ enzyme concentrate are standard ingredients used in the enzyme industry. All the raw materials conform to the specifications of the Food Chemical Codex, 6th edition (FCC 2008), except for those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal requirements have been made in line with FCC requirements and acceptability of use for food enzyme

production. Danisco uses a supplier quality program to qualify and approve suppliers. Raw materials are purchased only from approved suppliers and are verified upon receipt.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

4.2 Fermentation

KLM3´ is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Appendix B. The fermentation is an aerobic process and requires continuous addition of air to the fermenter. All equipment is carefully designed, constructed, operated, cleaned and maintained so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are conducted periodically to ensure absence of foreign microorganisms and confirm production strain identity.

The fermentation process consists of three operations: laboratory propagation of the culture, seed fermentation and primary fermentation. These processes, except for the laboratory propagation are carried out in sealed vessels carefully designed to prevent both the release of the production organism and/or the entry of other microorganisms.

A new lyophilized stock culture vial of the *B. licheniformis* production organism is used to initiate the production of each batch. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

The fermentation media is sterilised at 121°C for at least 20 minutes. The medium is sampled for microbiological testing prior to inoculation. The fermentation takes place at controlled temperatures.

All stages of the production process are controlled to ensure that the final product conforms to specifications. The culture liquid is sampled at intervals during fermentation for microbiological and enzyme activity tests. Operational parameters such as temperature, pH, air flow, agitation and oxygen content are monitored and controlled to desired values/ranges throughout the fermentation. In addition, at all stages, microbial growth is checked for correct morphological development of the microorganism and for the lack of contamination. Once the fermentation is completed, the fermentation broth is transferred to processing tanks.

4.3 Recovery

The purpose of the recovery process is to separate the biomass, purify, concentrate, and stabilise the desired enzyme, i.e. KLM3´.

Separation of the cell debris from the liquid from the fermentation broth is achieved by either filtration or centrifugation, or a combination of both. Exactly which cell separation technique is used is dependent upon the manufacturing site. The broth may be treated with flocculating agents to maximize separation and is then fed into the filter or the centrifuge. The relatively solids free stream then passes a polishing filter to further clarify the liquid and achieve clear, cell-free filtrate.

The liquid containing the enzyme is concentrated via ultrafiltration, which removes low molecular weight compounds. Diafiltration may follow ultrafiltration to help reach the activity target, remove colour and smaller particles, and carbon treatment may additionally be used to reduce colour. The final recovery step is a polish filtration using either microfiltration membranes, fine filtration aids such as diatomaceous earth or sterile filtration pads.

The ultrafiltered concentrate is then dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier, or stabilised by e.g. glycerol to produce a liquid product.

A manufacturing flow sheet is found in Appendix A6.

4.4 Formulation

The ultrafiltrated concentrate is then formulated and analysed in accordance with the general specifications for enzyme preparations used in food processing as established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) and the FCC.

Full details on raw materials and formulation ingredients used in the production of the enzyme can be found in Appendix E. Note that this information is proprietary and "**Confidential Commercial Information**" status is requested.

5 Specification for identity and purity

5.1 Purity criteria

Appropriate GMP controls and processes are used in the manufacture of KLM3´ to ensure that the finished product does not contain any impurities of a hazardous or toxic nature. The specification for impurities and microbial limits for the KLM3´ product can be found in Appendix A4. Certificates of Analysis for three lots of product are given in Appendix A5.

The specifications for the KLM3´ enzyme preparation meet or exceed the requirements for enzyme preparations as set forth in the Food Chemical Codex, 6th Edition (2008) (Appendix A7) and by the Joint FAO/WHO Expert Committee on Food additives (JECFA 2006) (Appendix A8).

5.2 Allergens

An allergen declaration of the enzyme concentrate can be found in Appendix A9.

5.3 Particle size analyses

This section is not applicable due to the liquid form of the enzyme.

6 References

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Aunstrup, K., Andersen, O., Falch, E. A., and Nielsen, T. K. 1979. Production of Microbial Enzymes in Microbial Technology, 2nd ed., Volume 1. Eds. Peppler, H.J., and Perlman, D., Chapter 9, pp. 282-309.

Enzyme Applications in Encyclopedia of Chemical Technology, 4th edition. 1994. Kroschwitz, J.I., Volume 9, pp. 567-620.

Food Chemicals Codex (FCC) 6th Edition. 2008. US Pharmacopeia, Rockville, Maryland.

JECFA (Joint FAO/WHO Expert Committee on Food Additives) 2006. General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

Appendix A1 : EC NUMBER

Source: IUBMB / **http://www.chem.qmul.ac.uk/iubmb/**

Accepted name: phosphatidylcholine—sterol *O*-acyltransferase

Reaction: phosphatidylcholine + a sterol = 1-acylglycerophosphocholine + a sterol ester

Other name(s): lecithin—cholesterol acyltransferase; phospholipid—cholesterol acyltransferase; LCAT (lecithin-cholesterol acyltransferase); lecithin:cholesterol acyltransferase; lysolecithin acyltransferase

Systematic name: phosphatidylcholine:sterol *O*-acyltransferase

Comments: Palmitoyl, oleoyl and linoleoyl residues can be transferred; a number of sterols, including cholesterol, can act as acceptors. The bacterial enzyme also catalyses the reactions of EC 3.1.1.4 phospholipase A_2 and EC 3.1.1.5 lysophospholipase.

Links to other databases: BRENDA, EXPASY, KEGG, CAS registry number: 9031-14-5

References:

1. Bartlett, K., Keat, M.J. and Mercer, E.I. Biosynthesis of sterol esters in *Phycomyces blakesleeanus. Phytochemistry* 13 (1974) 1107-1113.

2. Buckley, J.T., Halasa, L.N. and Macintyre, S. Purification and partial characterization of a bacterial phospholipid: cholesterol acyltransferase. *J. Biol. Chem.* 257 (1982) 3320-3325. [PMID: 7061477]

3. Glomset, J.A.J. The plasma lecithins:cholesterol acyltransferase reaction. *Lipid Res.* 9 (1968) 155-167. [PMID: 4868699]

4. Vahouny, G.V. and Tradwell, C.R. Enzymatic synthesis and hydrolysis of cholesterol esters. *Methods Biochem. Anal.* 16 (1968) 219-272. [PMID: 4877146]

[EC 2.3.1.43 created 1972, modified 1976]

Appendix A2 : CAS NUMBER

Source: SciFinder Database

Registry Number: 9031-14-5

Formula: Unspecified

CA Index Name: Acyltransferase, lecithin-cholesterol

Other Names: E.C. 2.3.1.43; LCAT; Lecithin-cholesterol acyltransferase

Class Identifier: Manual Registration

-- Resources --

References: ~2200

STN Files: CAPLUS, ADISNEWS, AGRICOLA, ANABSTR, BIOSIS, BIOTECHNO, CA, CABA, CIN, EMBASE, IFICDB, IFIPAT, IFIUDB, PROMT, TOXCENTER, USPAT2, USPATFULL

(Additional Information is available through STN International. Contact your information specialist, a local CAS representative, or the CAS Help Desk for Assistance)

Database: REGISTRY

Appendix A3 : Activity of the enzyme complex

Determination of phospholipase activity (LATU-K Assay)

Substrate:

0.6% L-α Phosphatidylcholine 95% Plant (Avanti #441601), 0.4% Triton-X 100 (Sigma X-100), and 5 mM $CaCl_2$ were dissolved in 0.05M HEPES buffer pH 7.

Assay procedure:

34 µl substrate was added to a cuvette, using a KoneLab automatic analyzer. At time T= 0 min, 4µl enzyme solution was added. Also a blank with water instead of enzyme was analyzed. The sample was mixed and incubated at 30°C for 10 minutes.

The free fatty acid content of sample was analyzed by using the NEFA C kit from WAKO GmbH.

Enzyme activity LATU pH 7 was calculated as micromole fatty acid produced per minute under assay conditions by using an internal standard enzyme for calibration.

Appendix A4 : Specification of the commercial product

Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Phospholipase	959W	Genencor Method	900-1100LATU/g
MICROBIOLOGICAL ANALYSIS			
Total Viable Count	800V	ISO 4833 - "Microbiology -General guidance for the enumeration of micro-organisms - colony count technique at 30°C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 50,000 CFU/g
Total Coliforms	810V	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Not more than 30 CFU/g
E. coli	819V	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Salmonella	832V	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	Negative/25g
Production strain	892V	Genencor Method	Negative by test
Antibacterial Activity	899V	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	Negative by test
OTHER ASSAYS			
Lead	603W-PB	AAS/ICP-AES method in Jecfa, Combined Compendium of Food Additive Specifications, Volume 4, Rome, 2006	Less than 5 mg/kg

Appendix A5: Certificates of analyses

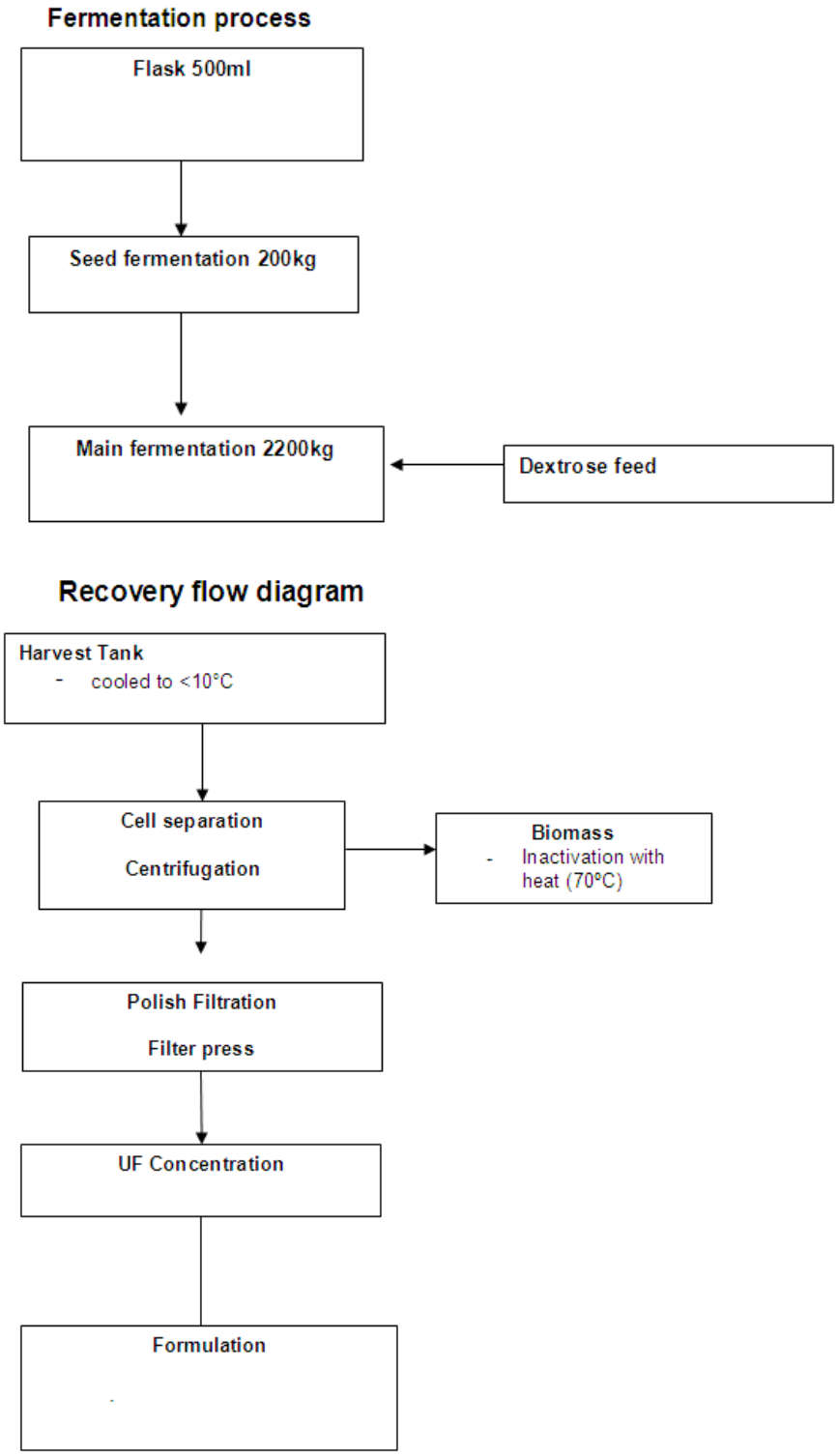
Property	Specification	Method reference	Lot 1600988976	Lot 1601002971	Lot 1601024294
Phospholipase	> 1000 LATU/g	Internal Method	1066 LATU/g	1230 LATU/g	1313 LATU/g
Total Viable Count	0 – 50000 CFU/ml	ISO 4833 - "Microbiology - General guidance for the enumeration of micro-organisms - colony count technique at 30C" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	92 CFU/ml	3 CFU/ml	<1 CFU/ml
Salmonella (cfu/25 ml)	Negative/25ml	Nordic Committee on Food Analysis; Salmonella Bacteria; Detection in Foods. No 71; 4th Edition; 1991 and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	NEG/25ml	NEG/25ml	NEG/25ml
Total Coliforms (cfu/ml)	0 - 30 CFU/ml	ISO 4832 - "General guidance for the enumeration of coliforms - colony count technique" and the FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	12 CFU/ml	12 CFU/ml	<1 CFU/ml
E. coli (cfu/25 ml)	Negative/25ml	ISO 7251 - Microbiology - "General Guidance for Enumeration of Presumptive *Escherichia coli* - Most Probable Number Technique" and FDA Bacteriological Analytical Manual; 8th Edition; AOAC International	NEG/25ml	NEG/25ml	NEG/25ml

Production Strain	Negative/ml	Proprietary	NEG/ml	NEG/ml	NEG/ml
Antimicrobial activity	Negative/ml	FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives; Geneva 1981; p317-318; Appendix A	NEG/ml	NEG/ml	NEG/ml
Lead (mg/kg)	0 – 5 mg/kg	AAS/ICP-AES method in Jecfa, Combined Compendium of Food Additive Specifications, Volume 4, Rome, 2006	<5 mg/kg	<5 mg/kg	<5 mg/kg

April 1, 2009
Date

Tracy Krawczyk
Divisional Quality Assurance

Appendix A6: Production process flowsheet



Appendix A7: Food Chemical Codex, 6th edition

Enzyme Preparations

DESCRIPTION

Enzyme Preparations used in food processing are derived from animal, plant, or microbial sources (see *Classification*, below). They may consist of whole cells, parts of cells, or cell-free extracts of the source used, and they may contain one active component or, more commonly, a mixture of several, as well as food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices. The individual preparations usually are named according to the substance to which they are applied, such as *Protease* or *Amylase*. Traditional names such as *Malt*, *Pepsin*, and *Rennet* also are used, however. The color of the preparations—which may be liquid, semiliquid, or dry—may vary from virtually colorless to dark brown. The active components consist of the biologically active proteins, which are sometimes conjugated with metals, carbohydrates, and/or lipids. Known molecular weights of the active components range from approximately 12,000 to several hundred thousand. The activity of enzyme preparations is measured according to the reaction catalyzed by individual enzymes (see below) and is usually expressed in activity units per unit weight of the preparation. In commercial practice (but not for *Food Chemicals Codex* purposes), the activity of the product is sometimes also given as the quantity of the preparation to be added to a given quantity of food to achieve the desired effect. Additional information relating to the nomenclature and the sources from which the active components are derived is provided under *Enzyme Assays*, Appendix V.

Function Enzyme (see discussion under *Classification*, below)

Packaging and Storage Store in well-closed containers in a cool, dry place.

IDENTIFICATION

Classification

- **ANIMAL-DERIVED PREPARATIONS**

Catalase, Bovine Liver: Produced as partially purified liquid or powdered extracts from bovine liver. Major active principle: *catalase*. Typical application: used in the manufacture of certain cheeses.

Chymotrypsin: Obtained from purified extracts of bovine or porcine pancreatic tissue. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymotrypsin*. Typical application: used in the hydrolysis of protein.

Lipase, Animal: Obtained from the edible forestomach tissue of calves, kids, or lambs; and from animal pancreatic tissue. Produced as purified edible tissue preparations or as aqueous extracts dispersible in water, but insoluble in alcohol. Major active principle: *lipase*. Typical applications: used in the manufacture of cheese and in the modification of lipids.

Lysozyme: Obtained from extracts of purified chicken egg whites. Generally prepared and used in the hydrochloride form as a white powder. Major active principle: *lysozyme*. Typical application: used as an antimicrobial in food processing.

Pancreatin: Obtained from porcine or bovine (ox) pancreatic tissue. Produced as a white to tan, water-soluble powder. Major active principles: (1) α-amylase; (2) protease; and (3) lipase. Typical applications: used in the preparation of precooked cereals, infant foods, and protein hydrolysates.

Pepsin: Obtained from the glandular layer of hog stomach. Produced as a white to light tan, water-soluble powder; amber paste; or clear, amber to brown, aqueous liquids. Major active principle: *pepsin*. Typical applications: used in the preparation of fishmeal and other protein hydrolysates and in the clotting of milk in the manufacture of cheese (in combination with rennet).

Phospholipase A_2: Obtained from porcine pancreatic tissue. Produced as a white to tan powder or pale to dark yellow liquid. Major active principle: *phospholipase* A_2. Typical application: used in the hydrolysis of lecithins.

Rennet, Bovine: Aqueous extracts made from the fourth stomach of bovines. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (pepsin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of sheep or goats.

Rennet, Calf: Aqueous extracts made from the fourth stomach of calves. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: *protease* (chymosin). Typical application: used in the manufacture of cheese. Similar preparations may be made from the fourth stomach of lambs or kids.

Trypsin: Obtained from purified extracts of porcine or bovine pancreas. Produced as white to tan, amorphous powders soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *trypsin*. Typical applications: used in baking, in the tenderizing of meat, and in the production of protein hydrolysates.

- **PLANT-DERIVED PREPARATIONS**

Amylase: Obtained from extraction of ungerminated barley. Produced as a clear, amber to dark brown liquid or a white to tan powder. Major active principle: β-*amylase*. Typical applications: used in the production of alcoholic beverages and sugar syrups.

Bromelain: The purified proteolytic substance derived from the pineapples *Ananas comosus* and *Ananas bracteatus* L. (Fam. Bromeliaceae). Produced as a white to light tan, amorphous powder soluble in water (the solution is usually colorless to light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *bromelain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, in the production of protein hydrolysates, and in baking.

Ficin: The purified proteolytic substance derived from the latex of *Ficus* sp. (Fam. Moraceae), which includes a variety of tropical fig trees. Produced as a white to off-white powder completely soluble in water. (Liquid fig latex concentrates are light to dark brown.) Major active principle: *ficin*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, and in the conditioning of dough in baking.

Malt: The product of the controlled germination of barley. Produced as a clear amber to dark brown liquid preparation or as a white to tan powder. Major active principles: (1) α-*amylase* and (2) β-*amylase*. Typical applications: used in baking, in the manufacture of alcoholic beverages and of syrups.

Papain: The purified proteolytic substance derived from the fruit of the papaya *Carica papaya* L. (Fam. Caricaceae). Produced as a white to light tan, amorphous powder or a liquid soluble in water (the solution is usually colorless or light yellow and somewhat opalescent), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *papain* and (2) *chymopapain*. Typical applications: used in the chillproofing of beer, in the tenderizing of meat, in the preparation of precooked cereals, and in the production of protein hydrolysates.

- **MICROBIALLY-DERIVED PREPARATIONS**

α-Acetolactatedecarboxylase: (*Bacillus subtilis* containing a *Bacillus brevis* gene) Produced as a brown liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually a light yellow to brown). Major active principle: *decarboxylase*. Typical application: used in the preparation of beer.

Aminopeptidase, Leucine: (*Aspergillus niger* var., *Aspergillus oryzae* var., and other microbial species) Produced as a light tan to brown powder or as a brown liquid by controlled fermentation using *Aspergillus niger* var., *Aspergillus oryzae* var., or other microbial species. The powder is soluble in water (the solution is usually light yellow to brown). Major active principles: (1) *aminopeptidase*, (2) *protease*, and (3) *carboxypeptidase* activities in varying amounts. Typical applications: used in the preparation of protein hydrolysates and in the development of flavors in processed foods.

Carbohydrase: (*Aspergillus niger* var., including *Aspergillus aculeatus*) Produced as an off-white to tan powder or a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. (including *Aspergillus aculeatus*). Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *pectinase* (a mixture of enzymes, including *pectin depolymerase, pectin methyl esterase*, *pectin lyase*, and *pectate lyase*), (3) *cellulase*, (4) *glucoamylase* (amyloglucosidase), (5) *amylo-1,6-glucosidase*, (6) *hemicellulase* (a mixture of enzymes, including *poly(galacturonate) hydrolase, arabinosidase, mannosidase, mannanase*, and *xylanase*), (7) *lactase*, (8) β-*glucanase*, (9) β-D-*glucosidase*, (10) *pentosanase*, and (11) α-*galactosidase*. Typical applications: used in the preparation of starch syrups and dextrose, alcohol, beer, ale, fruit juices, chocolate syrups, bakery products, liquid coffee, wine, dairy products, cereals, and spice and flavor extracts.

Carbohydrase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) *glucoamylase* (amyloglucosidase), and (3) *lactase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, ale, bakery products, and dairy products.

Carbohydrase: (*Bacillus acidopullulyticus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Bacillus acidopullulyticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *pullulanase*. Typical applications: used in the hydrolysis of amylopectins and other branched polysaccharides.

Carbohydrase: (*Bacillus stearothermophilus*) Produced as an off-white to tan powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus stearothermophilus*. Soluble in water, but practically insoluble in alcohol, in ether, and in chloroform. Major active principle: α*-amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, and bakery products.

Carbohydrase: (*Candida pseudotropicalis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Candida pseudotropicalis*. Soluble in water (the solution is usually light yellow to dark brown) but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Kluyveromyces marxianus* var. *lactis*) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Kluyveromyces marxianus* var. *lactis*. Soluble in water (the solution is usually light yellow to dark brown), but insoluble in alcohol, in chloroform, and in ether. Major active principle: *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: (*Mortierella vinaceae* var. *raffinoseutilizer*) Produced as an off-white to tan powder or as pellets by controlled fermentation using *Mortierella vinaceae* var. *raffinoseutilizer*. Soluble in water (pellets may be insoluble in water), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α*-galactosidase*. Typical application: used in the production of sugar from sugar beets.

Carbohydrase: (*Rhizopus niveus*) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using *Rhizopus niveus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α*-amylase* and (2) *glucoamylase*. Typical application: used in the hydrolysis of starch.

Carbohydrase: (*Rhizopus oryzae* var.) Produced as a powder or a liquid by controlled fermentation using *Rhizopus oryzae* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α*-amylase*, (2) *pectinase*, and (3) *glucoamylase* (amyloglucosidase). Typical applications: used in the preparation of starch syrups and fruit juices, vegetable purees, and juices and in the manufacture of cheese.

Carbohydrase: (*Saccharomyces* species) Produced as a white to tan, amorphous powder by controlled fermentation using a number of species of *Saccharomyces* traditionally used in the manufacture of food. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform,

and in ether. Major active principles: (1) *invertase* and (2) *lactase*. Typical applications: used in the manufacture of candy and ice cream and in the modification of dairy products.

Carbohydrase: [(*Trichoderma longibrachiatum* var.) (formerly *reesei*)] Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Trichoderma longibrachiatum* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *cellulase*, (2) β-*glucanase*, (3) β-D-*glucosidase*, (4) *hemicellulase*, and (5) *pentosanase*. Typical applications: used in the preparation of fruit juices, wine, vegetable oils, beer, and baked goods.

Carbohydrase: (*Bacillus subtilis* containing a *Bacillus megaterium* α-*amylase* gene) Produced as an off-white to brown, amorphous powder or liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: α-*amylase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, and dextrose.

Carbohydrase (*Bacillus subtilis* containing a *Bacillus stearothermophilus* α-amylase gene) Produced as an off-white to brown, amorphous powder or a liquid by controlled fermentation using the modified *Bacillus subtilis*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: maltogenic *amylase*. Typical applications: used in the preparation of starch syrups, dextrose, alcohol, beer, and baked goods.

Carbohydrase and Protease, Mixed: (*Bacillus licheniformis* var.) Produced as an off-white to brown, amorphous powder or as a liquid by controlled fermentation using *Bacillus licheniformis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase* and (2) *protease*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, fishmeal, and protein hydrolysates.

Carbohydrase and Protease, Mixed: (*Bacillus subtilis* var. including *Bacillus amyloliquefaciens*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Bacillus subtilis* var. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) α-*amylase*, (2) β-*glucanase*, (3) *protease*, and (4) *pentosanase*. Typical applications: used in the preparation of starch syrups, alcohol, beer, dextrose, bakery products, and fishmeal, in the tenderizing of meat, and in the preparation of protein hydrolysates.

Catalase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually tan to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical applications: used in the manufacture of cheese, egg products, and soft drinks.

Catalase: (*Micrococcus lysodeikticus*) Produced by controlled fermentation using *Micrococcus lysodeikticus*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *catalase*. Typical application: used in the manufacture of cheese, egg products, and soft drinks.

Chymosin: (*Aspergillus niger* var. *awamori, Escherichia coli K-12*, and *Kluyveromyces marxianus*, each microorganism containing a calf *prochymosin* gene) Produced as a white to tan, amorphous powder or as a light yellow to brown liquid by controlled fermentation using the above-named genetically modified microorganisms. The powder is soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *chymosin*. Typical application: used in the manufacture of cheese and in the preparation of milk-based desserts.

Glucose Isomerase: (*Actinoplanes missouriensis, Bacillus coagulans, Streptomyces olivaceus, Streptomyces olivochromogenes, Microbacterium arborescens, Streptomyces rubiginosus* var., or *Streptomyces murinus*) Produced as an off-white to tan, brown, or pink amorphous powder, granules, or liquid by controlled fermentation using any of the above-named organisms. The products may be soluble in water, but practically insoluble in alcohol, in chloroform, and in ether; or if immobilized, may be insoluble in water and partially soluble in alcohol, in chloroform, and in ether. Major active principle: *glucose* (or *xylose*) *isomerase*. Typical applications: used in the manufacture of high-fructose corn syrup and other fructose starch syrups.

Glucose Oxidase: (*Aspergillus niger* var.) Produced as a yellow to brown solution or as a yellow to tan or off-white powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow to brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *glucose oxidase* and (2) *catalase*. Typical applications: used in the removal of sugar from liquid eggs and in the deoxygenation of citrus beverages.

Lipase: (*Aspergillus niger* var.) Produced as an off-white to tan, amorphous powder by controlled fermentation using *Aspergillus niger* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical application: used in the hydrolysis of lipids (e.g., fish oil concentrates and cereal-derived lipids).

Lipase: (*Aspergillus oryzae* var.) Produced as an off-white to tan, amorphous powder or a liquid by controlled fermentation using *Aspergillus oryzae* var. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids (e.g., fish oil concentrates) and in the manufacture of cheese and cheese flavors.

Lipase: (*Candida rugosa;* formerly *Candida cylindracea*) Produced as an off-white to tan powder by controlled fermentation using *Candida rugosa*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of dairy products and confectionery goods, and in the development of flavor in processed foods.

Lipase: [*Rhizomucor (Mucor) miehei*] Produced as an off-white to tan powder or as a liquid by controlled fermentation using *Rhizomucor miehei*. Soluble in water (the solution is usually light yellow to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *lipase*. Typical applications: used in the hydrolysis of lipids, in the manufacture of cheese, and in the removal of haze in fruit juices.

Phytase: (*Aspergillus niger* var.) Produced as an off-white to brown powder or as a tan to dark brown liquid by controlled fermentation using *Aspergillus niger* var. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principles: (1) *3-phytase* and (2) *acid phosphatase*. Typical applications: used in the production of soy protein isolate and in the removal of phytic acid from plant materials.

Protease: (*Aspergillus niger* var.) Produced by controlled fermentation using *Aspergillus niger* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the production of protein hydrolysates.

Protease: (*Aspergillus oryzae* var.) Produced by controlled fermentation using *Aspergillus oryzae* var. The purified enzyme occurs as an off-white to tan, amorphous powder. Soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical applications: used in the chillproofing of beer, in the production of bakery products, in the tenderizing of meat, in the production of protein hydrolysates, and in the development of flavor in processed foods.

Rennet, Microbial: (nonpathogenic strain of *Bacillus cereus*) Produced as a white to tan, amorphous powder or a light yellow to dark brown liquid by controlled fermentation using *Bacillus cereus*. Soluble in water, but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: (*Endothia parasitica*) Produced as an off-white to tan, amorphous powder or as a liquid by controlled fermentation using nonpathogenic strains of *Endothia parasitica*. The powder is soluble in water (the solution is usually tan to dark brown), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Rennet, Microbial: [*Rhizomucor (Mucor)* sp.] Produced as a white to tan, amorphous powder by controlled fermentation using *Rhizomucor miehei*, or *pusillus* var. Lindt. The powder is soluble in water (the solution is usually light yellow), but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *protease*. Typical application: used in the manufacture of cheese.

Transglutaminase: (*Streptoverticillium mobaraense* var.) Produced as an off-white to weak yellow-brown, amorphous powder by controlled fermentation using *Streptoverticillium mobaraense* var. Soluble in water but practically insoluble in alcohol, in chloroform, and in ether. Major active principle: *transglutaminase*. Typical applications: used in the processing of meat, poultry, and seafood; production of yogurt, certain

cheeses, and frozen desserts; and manufacture of pasta products and noodles, baked goods, meat analogs, ready-to-eat cereals, and other grain-based foods.

- **REACTIONS CATALYZED**

[NOTE: The reactions catalyzed by any given active component are essentially the same, regardless of the source from which that component is derived.]

α-Acetolactatedecarboxylase: Decarboxylation of α-cetolactate to acetoin

Aminopeptidase, Leucine: Hydrolysis of *N*-terminal amino acid, which is preferably leucine, but may be other amino acids, from proteins and oligopeptides, yielding free amino acids and oligopeptides of lower molecular weight

α-Amylase: Endohydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding dextrins and oligo- and monosaccharides

β-Amylase: Hydrolysis of α-1,4-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding maltose and betalimit dextrins

Bromelain: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

Catalase: $2H_2O_2 \leftrightarrow O_2 + 2H_2O$

Cellulase: Hydrolysis of β-1,4-glucan bonds in such polysaccharides as cellulose, yielding β-dextrins

Chymosin (calf and fermentation derived): Cleaves a single bond in kappa casein

Ficin: Hydrolysis of polypeptides, amides, and esters (especially at bonds involving basic amino acids, leucine, or glycine), yielding peptides of lower molecular weight

α-Galactosidase: Hydrolysis of terminal nonreducing α-D-galactose residues in α-D-galactosides

β-Glucanase: Hydrolysis of β-1,3- and β-1,4-linkages in β-D-glucans, yielding oligosaccharides and glucose

Glucoamylase (amyloglucosidase): Hydrolysis of terminal α-1,4- and α-1,6-glucan bonds in polysaccharides (starch, glycogen, etc.), yielding glucose (dextrose)

Glucose Isomerase (xylose isomerase): Isomerization of glucose to fructose, and xylose to xylulose

Glucose Oxidase: β-D-glucose + $O_2 \leftrightarrow$ D-glucono-δ-lactone + H_2O_2

β-D-Glucosidase: Hydrolysis of terminal, nonreducing β-D-glucose residues with the release of β-D-glucose

Hemicellulase: Hydrolysis of β-1,4-glucans, α-L-arabinosides, β-D-mannosides, 1,3-β-D-xylans, and other polysaccharides, yielding polysaccharides of lower molecular weight

Invertase (β-fructofuranosidase): Hydrolysis of sucrose to a mixture of glucose and fructose (invert sugar)

Lactase (β-galactosidase): Hydrolysis of lactose to a mixture of glucose and galactose

Lysozyme: Hydrolysis of cell-wall polysaccharides of various bacterial species leading to the breakdown of the cell wall most often in Gram-positive bacteria

Maltogenic Amylase: Hydrolysis of α-1,4-glucan bonds

Lipase: Hydrolysis of triglycerides of simple fatty acids, yielding mono- and diglycerides, glycerol, and free fatty acids

Pancreatin:

α-Amylase: Hydrolysis of α-1,4-glucan bonds

Protease: Hydrolysis of proteins and polypepticles

Lipase: Hydrolysis of triglycerides of simple fatty acids

Pectinase:

Pectate lyase: Hydrolysis of pectate to oligosaccharides

Pectin depolymerase: Hydrolysis of 1,4 galacturonide bonds

Pectin lyase: Hydrolysis of oligosaccharides formed by pectate lyase

Pectinesterase: Demethylation of pectin

Pepsin: Hydrolysis of polypeptides, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues, yielding peptides of lower molecular weight

Phospholipase A_2: Hydrolysis of lecithins and phosphatidylcholine, producing fatty acid anions

Phytase:

3-Phytase: *myo*-Inositol hexakisphosphate + H_2O ↔ 1,2,4,5,6-pentakisphosphate + orthophosphate

Acid Phosphatase: Orthophosphate monoester + H_2O ↔ an alcohol + orthophosphate

Protease (generic): Hydrolysis of polypeptides, yielding peptides of lower molecular weight

Pullulanase: Hydrolysis of 1,6-α-D-glycosidic bonds on amylopectin and glycogen and in α-and β-limit dextrins, yielding linear polysaccharides

Rennet (bovine and calf): Hydrolysis of polypeptides; specificity may be similar to pepsin

Transglutaminase: Binding of proteins

Trypsin: Hydrolysis of polypeptides, amides, and esters at bonds involving the carboxyl groups of L-arginine and L-lysine, yielding peptides of lower molecular weight

ASSAY

- **PROCEDURE**

Analysis: The following procedures, which are included under *Enzyme Assays*, Appendix V, are provided for application as necessary in determining compliance with the declared representations for enzyme activity[1]: Acid Phosphatase Activity, α-Amylase Activity (Nonbacterial); Bacterial α-Amylase Activity (BAU); Catalase Activity; Cellulase Activity; Chymotrypsin Activity; Diastase Activity (Diastatic Power); α-Galactosidase Activity, β-Glucanase Activity; Glucoamylase Activity (Amyloglucosidase Activity);

Glucose Isomerase Activity; Glucose Oxidase Activity; β-D-Glucosidase Activity; Hemicellulase Activity; Invertase Activity; Lactase (Neutral) (β-Galactosidase) Activity; Lactase (Acid) (β-Galactosidase) Activity; Lipase Activity; Lipase/Esterase (Forestomach) Activity; Maltogenic Amylase Activity; Milk-Clotting Activity; Pancreatin Activity; Pepsin Activity; Phospholipase Activity; Phytase Activity; Plant Proteolytic Activity; Proteolytic Activity, Bacterial (PC); Proteolytic Activity, Fungal (HUT); Proteolytic Activity, Fungal (SAP); Pullulanase Activity; and Trypsin Activity.

Acceptance criteria: NLT 85.0% and NMT 115.0% of the declared units of enzyme activity

IMPURITIES

- **LEAD,** *Lead Limit Test*, Appendix IIIB

Control: 5 µg Pb (5 mL of *Diluted Standard Lead Solution*)

Acceptance criterion: NMT 5 mg/kg

SPECIFIC TESTS

- **MICROBIAL LIMITS**

[NOTE: Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at www.cfsan.fda.gov/.]

Acceptance criteria:

Coliforms: NMT 30 CFU/g

Salmonella: Negative in 25 g

OTHER REQUIREMENTS

Change to read:

Enzyme preparations are produced in accordance with good manufacturing practices. Regardless of the source of derivation, they should cause no increase in the total microbial count in the treated food over the level accepted for the respective food.

Animal tissues used to produce enzymes must comply with the applicable U.S. meat inspection requirements and must be handled in accordance with good hygienic practices.

Plant material used to produce enzymes or culture media used to grow microorganisms consist of components that leave no residues harmful to health in the finished food under normal conditions of use.

▲Preparations derived from microbial sources shall be obtained using a pure culture fermentation of a non-pathogenic and non-toxigenic strain and are produced by methods and under culture conditions that ensure a controlled fermentation, thus preventing the introduction of microorganisms that could be the source of toxic materials and other undesirable substances.▲ *FCC 6*

The carriers, diluents, and processing aids used to produce the enzyme preparations shall be substances that are acceptable for general use in foods, including water and substances that are insoluble in foods but removed from the foods after processing.

Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants.

[1] Because of the varied conditions under which pectinases are employed, and because laboratory hydrolysis of a purified pectin substrate does not correlate with results observed with the natural substrates under use conditions, pectinase suppliers and users should develop their own assay procedures that would relate to the specific application under consideration.

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
Monograph	Kristie Bowman Senior Scientific Associate 1-301-816-8356	(FI07) Food Ingredients Expert Committee

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Appendix A8 : General specifications for enzyme preparations used in food processing

General Specifications and Considerations for Enzyme Preparations Used in Food Processing

The following general specifications were prepared by the Committee at its sixty-seventh meeting (2006) for publication in FAO JECFA Monographs 3 (2006), superseding the general specifications prepared at the fifty-seventh meeting (*1*) and published in FAO JECFA Monographs 1 (*2*). These specifications were originally prepared by the Committee at its twenty-fifth meeting (*3*) and published in FAO Food and Nutrition Papers No. 19 and No. 31/2 (*4,5*). Subsequent revisions were made by the Committee at its thirty-fifth meeting and published in FAO Food and Nutrition Paper No. 52 (*6*). Additional amendments were made at the fifty-first meeting and published in FAO Food and Nutrition Paper No. 52 Add. 6 *(7*), and at the fifty-third meeting *(8*) and partially published in FAO Food and Nutrition Paper No. 52 Add. 7 *(9)*.

Classification and nomenclature of enzymes

Enzymes are proteins that catalyse chemical reactions. The Enzyme Commission of the International Union of Biochemistry and Molecular Biology (formerly the International Union of Biochemistry) classified enzymes into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (*10*). Based on the type of reaction catalysed, enzymes are assigned to one of these classes and given an Enzyme Commission (EC) number, a systematic name, and a common name. Other names are also provided, if available. Enzymes used in food processing are often referred to by their common or traditional names such as protease, amylase, malt, or rennet. For enzymes derived from microorganisms, the name of the source microorganism is usually specified, for example, "α-amylase from *Bacillus subtilis*." For enzymes derived from microorganisms modified by using recombinant DNA techniques (referred to as recombinant-DNA microorganisms or genetically modified microorganisms), the names of both the enzyme source (donor organism) and the production microorganism are provided, for example, "α-amylase from *Bacillus licheniformis* expressed in *Bacillus subtilis*."

Enzyme preparations

Enzymes are used in food processing as enzyme preparations. An enzyme preparation contains an active enzyme (in some instances a blend of two or more enzymes) and intentionally added formulation ingredients such as diluents, stabilizing agents, and preserving agents. The formulation ingredients may include water, salt, sucrose, sorbitol, dextrin, cellulose, or other suitable compounds. Enzyme preparations may also contain constituents of the source organism (i.e. an animal, plant, or microbial material from which an enzyme was isolated) and compounds derived from the manufacturing process, for example, the residues of the fermentation broth. Depending on the application, an enzyme preparation may be formulated as a liquid, semi-liquid or dried product. The colour of an enzyme preparation may vary from colourless to dark brown. Some enzymes are immobilized on solid support materials.

Active components

Enzyme preparations usually contain one principal enzyme that catalyses one specific reaction during food processing. For example, α-amylase catalyses the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related polysaccharides. However, some enzyme preparations contain a mixture of enzymes that catalyse two or more different reactions in food. Each principal enzyme present in an enzyme preparation is characterized by its systematic name, common name, and EC number. The activity of each enzyme is measured using an appropriate assay and expressed in defined activity units per weight (or volume) of the preparation.

Source materials

Enzymes used in food processing are derived from animal, plant, and microbial sources. Animal tissues used for the preparation of enzymes should comply with meat inspection requirements and be handled in accordance with good hygienic practice.

Plant material and microorganisms used in the production of enzyme preparations should not leave any residues harmful to health in the processed finished food under normal conditions of use.

Microbial strains used in the production of enzyme preparations may be native strains or mutant strains derived from native strains by the processes of serial culture and selection or mutagenesis and selection or by the application of recombinant DNA technology. Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis (*11–15*). Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species.

Microbial production strains should be taxonomically and genetically characterized and identified by a strain number or other designation. The strain identity may be included in individual specifications, if appropriate. The strains should be maintained under conditions that ensure the absence of genetic drift and, when used in the production of enzyme preparations, should be subjected to methods and culture conditions that are applied consistently and reproducibly from batch to batch. Such conditions should prevent the introduction of microorganisms that could be the source of toxic and other undesirable substances. Culture media used for the growth of microbial sources should consist of components that leave no residues harmful to health in the processed finished food under normal conditions of use.

Enzyme preparations should be produced in accordance with good food manufacturing practice and cause no increase in the total microbial count in the treated food over the level considered to be acceptable for the respective food.

Substances used in processing and formulation

Substances used in processing and formulation of enzyme preparations should be suitable for their intended uses.

In the case of immobilized enzyme preparations, leakage of active enzymes, support materials, crosslinking agents and/or other substances used in immobilization should be kept within acceptable limits established in the individual specifications.

To distinguish the proportion of the enzyme preparation derived from the source material and manufacturing process from that contributed by intentionally added formulation ingredients, the content of total organic solids (TOS) is calculated as follows:

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

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients.

Purity

Lead:
Not more than 5 mg/kg.
Determine using an atomic absorption spectroscopy/inductively coupled atomic-emission spectroscopy (AAS/ICP-AES) technique appropriate to the specified level. The selection of the sample size and the

method of sample preparation may be based on the principles described in the *Compendium of Food Additive Specifications*, Volume 4.

Microbiological criteria:
Salmonella species: absent in 25 g of sample
Total coliforms: not more than 30 per gram
Escherichia coli: absent in 25 g of sample
Determine using procedures described in Volume 4.

Antimicrobial activity:
Absent in preparations from microbial sources.

Other considerations

Safety assessment of food enzyme preparations has been addressed in a number of publications and documents. Pariza & Foster (*11*) proposed a decision tree for determining the safety of microbial enzyme preparations. Pariza & Johnson (*16*) subsequently updated this decision tree and included information on enzyme preparations derived from recombinant-DNA microorganisms. The Scientific Committee on Food (*17*) issued guidelines for the presentation of data on food enzymes. The document includes a discussion on enzymes from genetically modified organisms including microorganisms, plants, and animals. Several international organizations, government agencies, and expert groups have also published discussion papers or guidelines that address safety assessment of food and food ingredients derived from recombinant-DNA plants and microorganisms (*18–28*). Certain information in these documents may be applicable to enzyme preparations derived from recombinant sources.

An overall safety assessment of each enzyme preparation intended for use in food processing should be performed. This assessment should include an evaluation of the safety of the production organism, the enzyme component, side activities, the manufacturing process, and the consideration of dietary exposure. Evaluation of the enzyme component should include considerations of its potential to cause an allergic reaction. For enzyme preparations from recombinant-DNA microorganisms, the following should also be considered:

1. The genetic material introduced into and remaining in the production microorganism should be characterized and evaluated for function and safety, including evidence that it does not contain genes encoding known virulence factors, protein toxins, and enzymes involved in the synthesis of mycotoxins or other toxic or undesirable substances.

2. Recombinant-DNA production microorganisms might contain genes encoding proteins that inactivate clinically useful antibiotics. Enzyme preparations derived from such microorganisms should contain neither antibiotic inactivating proteins at concentrations that would interfere with antibiotic treatment nor transformable DNA that could potentially contribute to the spread of antibiotic resistance.

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Appendix A9 : Allergen declaration on the enzyme liquid concentrate

GENENCOR®
A Danisco Division

Allergenic Ingredients

Product	**KLM 3 Prime Concentrate**
Article-no.	**C14039**

The table below indicates the presence (as added component) of the following allergens and products thereof *. **Unless otherwise noted, the following listed allergens and products thereof have been used in the recovery process or in the formulation of an enzyme product:**

YES	NO	Allergens	Description of components
	X	Wheat	
(X)		Other cereals containing gluten	Glucose, (used in fermentation)**
	X	Crustaceans	
	X	Eggs	
	X	Fish	
	X	Peanuts	
(X)		Soybeans	Soy bean grits (used in fermentation)**
	X	Milk (including lactose)	
	X	Nuts	
	X	Celery	
	X	Mustard	
	X	Sesame Seeds	
	X	Sulphur dioxide and sulfites >10mg/kg	
	X	Lupine and products thereof	
	X	Mollusk and products thereof	

*Local legislation has always to be consulted as allergen labeling requirements may vary from country to country.

**Danisco has determined that fermentation nutrients are outside the scope of US and EU food allergen labeling requirements[1, 2].

1) Position paper sent by the Enzyme Technical Association to the FDA on September 12, 2005 (see, http://enzymetechnicalassoc.org/Allergen%20psn%20paper-2.pdf)

2) Summarized in the position paper of the Association of Manufacturers and Formulators of Enzyme Products: http://www.amfep.org/documents/AmfepstatementScopeAllergyLabellingDirfinal_000.pdf

Completed By: Summer Evert, Associate Toxicologist
Date: April 30, 2010

June 28, 2010

APPENDIX B: Safety

Contents:

Appendices B

B1	Certificates of analyses of the test articles
B2	Risk assessment for potential food allergenicity
B3	Analysis of safety based on Pariza-Johnson Decision Tree

1 Toxicity of the enzyme

To assess the safety of KLM3' enzyme preparation produced by *Bacillus licheniformis* the following tests were conducted:

Study	Test Item
Acute oral toxicity in rats – Fixed dose procedure	UF concentrate
Sub-chronic 13 week toxicity in the rat	UF concentrate
Ames mutagenicity study	Lyophilized UF concentrate
In vitro chromosomal aberration Study	Lyophilized UF concentrate
In vivo Mouse micronucleus study	Lyophilized UF concentrate

1.1 Test article description

To assess the safety of KLM3' in foods, different endpoints of toxicity were investigated at LAB Scantox (Denmark) and are evaluated and assessed in this document. The test material used in the acute and oral gavage studies was the UF concentrate from Lot No. 20068010 containing 30.40 mg total protein/ml (equivalent to 86.70 mg TOS/ml). The test material used in all genotoxicity studies was the lyophilized powder of the UF concentrate from Lot No. 20068010. Both the UF and lyophilized powder (88.9% dry matter) are fully described in the Certificate of Analyses (Appendix B1).

1.2 Toxicological tests

- **Acute oral toxicity in rats – Fixed dose procedure**

Test report 62123 from Scantox Laboratories dated September 2006 is attached in a separate binder.

Procedure:
The objective of this study is to assess the acute toxicity of KLM3' when administered as a single oral dose followed by a 14-day period of observation. The information is used for both hazard assessment and ranking purposes. The study was initiated with a sighting study using two dose levels - 300 and 600 mg total protein/kg – with one rat per dose level. The 600 mg/kg dose level was the maximum dose that can be given due to limitations of the dosing volume (maximum = 20 ml/kg bw) and total protein concentration (30.4 mg per ml). The main study was performed in four additional female rats given a dose of 600 mg total protein/kg bw.

This study was conducted according to OECD Guideline No. 420 (Acute oral toxicity – Fixed dose procedure) and in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1977).

Results:

No mortality was recorded in this study. Transient weight losses of 3 to 4 g were noted in 2/5 animals treated with 600 mg/kg (20 ml/kg). There were no overt signs of systemic toxicity throughout the 14-day observation period.

Evaluation:
Under the conditions of this study, the oral LD_{50} was >600 mg total protein/kg body weight (20 ml/kg of test article).

- **Bacterial Reverse Mutation Assay – Ames assay.**

Test report 62127 from Scantox Laboratories dated October 2006 is attached in a separate binder.

Procedure:
The objective of this assay is to assess the potential of KLM3' to induce point mutations (frame-shift and base-pair) in five strains of *Salmonella typhimurium*: TA 98, TA 100, TA 102, TA 1535 and TA 1537. The test material was tested both in the presence and absence of a metabolic activation system (Aroclor 1254-induced rat liver; S-9 mix). The tests were performed using the "treat and plate" method to avoid the possibility of interference from histidine in the test article. In the treat and plate method, various concentrations of KLM3' were mixed with a concentrated bacterial suspension and nutrient broth. These mixtures were incubated at 37°C under shaking for 3 hours. At the end of the 3-hour period, the bacteria were sedimented by centrifugation, the supernatant was removed and the bacteria were resuspended in 2 ml buffer. The cultures were then centrifuged, the supernatant removed and the bacteria resuspended a second time in buffer and top agar was added. The contents of each tube were mixed and spread on selective agar plates. The plates were then incubated for 72 hours at 37°C and then scored for revertants and viability.

A preliminary toxicity test was performed in strain TA 98. Subsequently, two independent main tests were performed with all 5 strains in both presence and absence of S-9 mix. Triplicate plates were used at each test point. Seven sequential dose levels of KLM3' were used in the main tests and ranged from 0.16 to 5000 µg/plate. All dose levels were expressed in terms of the weight of the freeze-dried sample of the test material. The highest dose level tested (5000 µg/plate) is the maximum required by the OECD guideline. The positive controls used for assays without S-9 mix were sodium azide, 2-nitrofluorene, 9-amino acridine and cumene hydroperoxide and the positive control used for assays with S-9 mix was 2-aminoanthracene.

This assay was conducted in accordance with OECD guideline No. 471 and complied with OECD Principles on GLP (as revised in 1997) and all subsequent OECD consensus documents.

Results:

A dose-related amount of insoluble material was observed on all plates. KLM3' was cytotoxic and the level of cytotoxicity varied between the tester strains, and between treatments in the presence and absence of S-9 mix. Reduced growth of the background lawn was seen in TA 1537 in the presence of S-9 mix. KLM3' was not toxic to TA 100 with S-9 mix and TA 98 with S9-mix. The dose levels (µg/plate) used were as follows:

	Main Test #1 (µg/plate)		**Main Test #2 (µg/plate)**	
Strains	Without S-9	With S-9 mix	Without S-9 mix	With S-9 mix
TA 102	1.6 – 1,600	5.0 – 5,000	0.16 – 160	5.0 – 5,000
TA 100	0.16 – 160	5.0 – 5,000	0.16 – 160	5.0 – 5,000
TA 98	0.16 – 160	5.0 – 5,000	0.16 – 160	5.0 – 5,000
TA 1537	0.16 – 160	5.0 – 5,000	0.16 – 160	5.0 – 5,000
TA 1535	1.6 – 1,600	5.0 – 5,000	0.16 – 160	5.0 – 5,000

5000 µg/plate = maximum required by OECD guideline.

No biologically or statistically significant increases in the number of revertant colonies were observed in any tester strain after treatment with KLM3' at any dose level either in the presence or absence of S-9 mix.

Statistical increases in the number of revertant colonies were noted with the positive controls in both the presence and absence of metabolic activation substantiating the sensitivity of the treat and plate assay and the efficacy of the metabolic activation mixture.

Evaluation
No biologically or statistically significant increases in the number of revertant colonies were noted after exposure to KLM3'.

Under the conditions of this assay, KLM3' has not shown any evidence of mutagenic activity in the Ames assay.

- ***In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes.**

Test report 62128 from Scantox Laboratories dated November 2006 is attached in a separate binder.

Procedure
The objective of this assay is to investigate the potential of KLM3' to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). KLM3' concentrate was mixed with cultures of human peripheral lymphocytes both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver; S-9 mix). This assay consisted of a preliminary toxicity (dose range finding) assay and two main tests. Five concentrations of KLM3' were used in the preliminary assay and at least 3 dose levels were then selected for the two main assays with the highest dose level clearly inducing a toxic effect (50% reduction in mitotic index). In the absence of

cytotoxicity, the highest dose selected would be 5000 µg/ml, as recommended by OECD guideline.

In the first main test, all cultures (with or without S-9 mix) were treated for 3 hours. In the second main test, cultures without S-9 mix were treated for 20 hours and those with S-9 mix for 3 hours. All cultures (with and without S-9 mix) were harvested 20 hours (1.5 normal cell cycles) after the start of treatment. Two hours prior to harvest, Demecolcine (0.1 µg/ml) was added to all cultures to arrest all cells at the metaphase-stage of mitosis. At the harvest time, all cultures were centrifuged and the supernatant discarded. The cell pellets were resuspended in a KCl solution, incubated for 10 minutes, centrifuged and the supernatant removed. The cells were then fixed on slides, stained and scored for chromosomal aberrations:

- Cytotoxicity was evaluated using the mitotic index (number of cells in mitosis/1000 cells examined. From these results, a dose level causing a decrease in mitotic index of 50% was selected as the highest dose in the main tests.
- Metaphase analysis (i.e. evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.
- Daunomycin C and cyclophosphamide were used as positive controls for cultures without S-9 mix and with S-9 mix, respectively.

This assay was conducted in accordance with OECD guideline No. 473 (*In vitro* Mammalian chromosome aberration test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

Results

The test article was unusually toxic to the lymphocytes *in vitro*. The highest test concentrations selected were:

First main test:

Without S-9 mix:	Highest dose = 256 µg/ml (3-hour exposure)
With S-9 mix:	Highest dose = 0.25 µg/ml (3-hour exposure)

Second main test:

Without S-9 mix:	Highest dose = 8.0 µg/ml (20-hour exposure)
With S-9 mix:	Highest dose = 0.0625 µg/ml (3-hour exposure)

KLM3' caused cytotoxicity and the highest dose levels tested in the first and second main tests in the absence of S-9 mix were 256 and 8 µg/ml, respectively. In the presence of S-9 mix, the highest doses selected were much lower (0.0625 to 0.25 µg/ml). These concentrations met the requirements of OECD 473 guideline for the highest concentration to be scored for aberrations (greater than 50% reduction in mitotic index). It is evident that cytotoxicity increased with duration of exposure (20-hr vs. 3 hr-exposure; in the absence of S-9 mix) and presence of metabolic activation.

At non-cytotoxic doses, no biologically or statistically significant increases in the frequency of metaphases with chromosomal aberrations were observed in cultures treated with KLM3' lyophilized powder both in the presence and absence of metabolic activation. Significant increases in aberrant metaphases were demonstrated with the positive controls.

Evaluation

Under the conditions of this test, KLM3' lyophilized powder did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation. However, KLM3' was extremely cytotoxic to the lymphocytes in cultures. The cytotoxic effect is related to the mechanism of action of an acyltransferase enzyme. Indeed, KLM3' effectiveness is based on its effects of transferring acyl groups from phospholipids and glycolipids to acceptors such as sterols, fatty alcohols and other smaller primary alcohols. Phospholipids are essential constituents of cell membranes and in the presence of KLM3'in an *in vitro* culture, a large proportion of the phospholipids are converted to lyso-phosphatidylcholine and lyso-phosphatidylethanolamine. The presence of appreciable amounts of lyso-derivatives in an *in vitro* situation is detrimental to the cell membrane leading to cell lysis.

To ascertain that cytotoxicity noted in this *in vitro* assay is limited to cells in culture, an *in vivo* mouse micronucleus test (described below) was initiated.

- ***In* vivo Mouse Micronucleus Test**

Test report 64145 from Scantox Laboratories dated December 2006 is attached in a separate binder.

Procedure

The objective of this study is to determine the potential of KLM3' given by the oral route to cause genotoxic effects resulting in the formation of micronuclei in erythrocytes in the bone marrow of treated mice. A measure of the genotoxic effect is obtained by comparing the frequency of micronucleated PCE (polychromatic erythrocytes) from the bone marrow of treated mice (stock Bom:NMRI from Taconic Europe, Denmark) with the corresponding control mice. A preliminary test was performed to identify the maximum dose level and was conducted with groups of 2 male and 2 female mice. The maximum dose level of 2000 mg/kg was given by oral gavage on two occasions separated by 24 hours. Body weight was recorded and the mice were killed 24 hours after the second treatment.

In the main assay, groups of 5 male mice each received 0 (vehicle), 500, 1000, or 2000 mg/kg/day of KLM3'on two occasions separated by 24 hours. The 2000 mg/kg dose level is the maximum dose required by the OECD guideline. A group receiving a single oral dose of cyclophosphamide (20 mg/kg) served as positive

control. All animals were killed 24 hours after the second treatment and bone marrow was collected from the femur. The bone marrow was suspended in an appropriate medium, centrifuged and the supernatant discarded. Smears of the cell pellet were made on glass slides. The following counts were made for each animal:

- Number of PCE per 1000 erythrocytes;
- Number of PCE with micronuclei in 2000 PCE; and
- Number of normochromatic erythrocytes (NCE) with micronuclei observed during the scoring of 1000 erythrocytes.

This assay was conducted in accordance with OECD guideline No. 474 (*In vivo* Mammalian Erythrocyte Micronucleus test) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

Results

In the preliminary test using groups of 2 males and 2 females, one female dosed at 2000 mg/kg was found dead at 1.5 hours after dosing on Day 1 and one male dosed at 1000 mg/kg lost 5 g in body weight between Day 1 and 2. The cause of death was probably related to dosing/gavaging error due to the presence of blood in the oral and nasal cavities.

The main test was performed using male mice only since effects observed in the preliminary test did not suggest a substantial difference in toxicity of the test article between the sexes. No adverse reactions to treatment and no biologically significant effects on body weights were observed in any mice. Small reductions in the mean percentage of PCE were observed at the 2000 mg/kg dose level compared to the vehicle control group (43.4% vs. 45.2%) but the effect was not considered as biologically significant due to large standard deviations noted in the treated groups. There were no effects on the number of micronuclei PCE in the KLM3' groups. The positive control produced a significant increase in micronuclei PCE substantiating the sensitivity and validity of the assay.

Evaluation

Under the conditions of this *in vivo* mouse micronucleus assay, evidence of cytotoxicity and genotoxicity was not demonstrated up to the maximum dose required by OECD guideline (i.e., 2000 mg/kg).

- **A 13-week Oral (Gavage) Toxicity Study in Rats**

Test report 62129 from Scantox Laboratories dated October 2006 is attached in a separate binder.

Procedure

The objective of this study is to investigate the potential of KLM3' to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (Taconic M&B, Denmark) of both sexes for 90 consecutive days. Groups of

10 rats/sex each were gavaged daily with 0 (sterile water containing 3% NaCl), 4.56, 13.68 or 41.00 mg total protein/kg body weight in a constant volume of 5 ml/kg body weight corresponding to 0, 13.0, 39.0 and 116.9 mg TOS/kg bw/day, respectively.

All animals were observed daily for mortality and signs of morbidity. Animals of the same sex were pair-housed in transparent polycarbonate cages with softwood sawdust as bedding and had access to water (via bottle) and feed *ad libitum*. All groups were housed under controlled temperature, humidity and lightning conditions. Body weight and feed consumption were recorded weekly. Ophthalmologic examination was performed on all animals prior to study initiation and in the control and high dose groups at study termination. Hematology was conducted on Day 0, 30, 60 and 90. A functional observation battery consisting of detailed clinical observation, reactivity to handling and stimuli, and motor activity examination was conducted during week 13 for the control and high dose rats. Clinical chemistry was evaluated at study termination prior to necropsy on all groups. After a thorough macroscopic examination, selected organs were removed, weighed and processed for future histopathologic examination. Microscopic examination was conducted on selected organs from control and high dose animals. If a questionable finding was noted, the microscopic examination would be extended to the low and mid dose groups.

This study was conducted in accordance with OECD guideline No. 408 (September 1998) and EPA Guideline OPPTS 870.3100 (August 1998) and complied with OECD Principles of GLP (as revised in 1997) and all subsequent OECD consensus documents.

Results

In Group 2 (low dose) a male was erroneously caged with a female during the first three-days of the study. Two extra females were added to Group 2 and these animals received KLM3' for two additional weeks after the main terminal kill. This deviation did not affect the integrity of the results.

There were 4 deaths recorded throughout the study and the mortality distribution is as follows:

Group 1 (control): No death

Group 2 (low dose): No death

Group 3 (mid dose): Three deaths:

One female was found dead on Day 12. Examination conducted at necropsy suggested a gavage-related accident (accumulation of red fluid in the lung).

One female was found dead on Day 29. Examination conducted at necropsy suggested a gavage-related accident (red discoloration of the lungs and pleuritis on the lungs).

One male was found dead on Day 73. Examination conducted at necropsy suggested a gavage-related accident (accumulation of red fluid in the lung).

Group 4 (high dose): One death

One male was found dead on Day 37. The animal was decomposed. Microscopic examination did not reveal any changes related to treatment or the dosing procedure. This death was not attributed to KLM3' due to its early occurrence (Day 37) and no additional mortality was noted in the high dose male and female groups.

One male (Group 2; low dose) was killed *in moribund* on Day 74 due to poor health (forced respiration, gasping for air, wheezing sound at respiration, and dehydration). Macroscopic examination revealed hemorrhage of the thymus. The poor health condition of the animal was not considered as treatment related due to its isolated incidence.

No clinical signs were seen that could be considered to be treatment related. There were no biological or statistical differences between the control and treated groups with respect to feed consumption, body weights, body weight gains, clinical chemistry, and ophthalmologic examinations. A statistically significant decrease in hemoglobin was noted in Group 4 males (high dose) on Days 35 and 36 but the value returned to normal by study termination. At study termination, a statistically significant increase in blood urea was noted in high dose males. As this was seen in one sex only, and with no clear dose response relationship, the increase was not considered to be attributable to treatment. No treatment related effects were noted in the functional observation battery test, macroscopic findings, and histopathologic examinations.

Evaluation

Although 4 animals were found dead and one low dose male was killed *in moribund*, these deaths could not be attributed to treatment since (1) dosing/gavage error accounted for 3 deaths, (2) the high dose male death occurred in the early phase of the study (Day 37), (3) lack of dose response relationship, (4) lack of clinical signs, and (5) lack of treatment-related macroscopic and microscopic findings. The increase in blood urea noted in high dose males at study termination was not attributable to treatment since the effect was seen in one sex and the values are still within the historical control range for clinical chemistry at Scantox laboratories (urea high dose males = 7.24 $\pm$ 0.54 mmol/L vs. Historical control urea males = 7.56 mmol/L).

Under the conditions of this assay, the NOAEL (no observed adverse effect level) is established at the highest dose tested (41.00 mg total protein/kg bw/day or 116.90 mg TOS/kg bw/day).

1.3 Safety assessment

According to the Directive of the Commission 93/21/EEC of April 27, 1993, KLM3' is non hazardous based on acute oral studies. In genotoxicity studies, KLM3' is not mutagenic, clastogenic or aneugenic. Daily oral administration of KLM3' up to and including a dose level of 41 mg total protein/kg bw/day or 116.90 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

- ***Identification of the NOAEL***

In the 90-day oral (gavage) study in rats (Scantox No. 62129), a NOAEL was established at 41 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day). The study was conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice and was designed based on OECD guideline No. 408. Since human exposure to KLM3' is through oral ingestion, selection of this NOAEL is thus appropriate.

No Observed Adverse Effect Level = 41 mg total protein/kg bw/day

2 Information on the source micro-organism

The function of the genetic modification is to produce the KLM3' glycerophospholipid-cholesterol acyltransferase (GCAT) enzyme of the donor strain *Aeromonas salmonicida* subsp. *salmonicida*, using a known safe *Bacillus licheniformis* host strain. During the strain construction, the new organism lost the capacity to sporulate due to deletion of a sporulation gene.

2.1 The production strain

The production organism of the KLM3´ enzyme preparation is *Bacillus licheniformis* strain GICC03265. It is derived by recombinant DNA methods from a strain of *Bacillus licheniformis* modified to express a synthetic KLM3´ gene DNA that has been modified at one amino acid and codon optimized for expression in *Bacillus licheniformis*. *Bacillus licheniformis* is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide. It also meets the criteria for a safe production microorganism as described by Pariza and Johnson (2001) and several expert groups (Pariza & Foster, 1983; IFBC, 1990; EU SCF, 1991; OECD, 1993; Berkowitz & Maryanski, 1989; FAO/WHO, 1996; Jonas et al., 1996) It contains the synthetic KLM3´ gene under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene.

2.2 The host

The host organism is *B. licheniformis* Bra7. *B. licheniformis* Bra7 was modified through deletion of several enzyme activities (proteases, amylase), a sporulation gene and the native chloramphenicol resistance genes to make it suitable for expression of KLM3'. This strain lineage has been used by Danisco as a host for the commercial production of a number of α-amylases for the starch liquefaction business since 1989, as well as for production of protease, pullulanase and xylanase. The strain has a sporulation frequency of less than 10^{-8} as determined by comparing the titer of the colony forming units (cfu) in the culture before and after heating at 85° C for 10 minutes. The strain, which has a history of safe large-scale fermentation, has been typed as *B. licheniformis* based on 16S rDNA gene sequencing and ribotyping.

2.3 The donor organism

The donor organism is the bacterium *Aeromonas salmonicida* subsp. *Salmonicida.* The strain was obtained from the American Type Culture Collection as ATCC # 14174. The gene which encodes the glycerophospholipid-cholesterol acyltransferase (KLM3´) enzyme of the donor strain *Aeromonas salmonicida* subsp. *salmonicida* was synthesized according to the known DNA sequence, modified at one amino acid position, asparagine at position 80 has been changed to aspartic acid, and codon optimized for expression in *B. licheniformis*

2.4 The vector

The vector used in this construction contains the following features: a temperature sensitive origin of replication (*ori* pE194, for replication in *Bacillus*), *ori* pBR322 (for amplification in *E. coli*), the pUB110 neomycin resistance gene (*neo*) for initial selection, and the native *B. licheniformis* chloramphenicol resistance gene (*cat*H) for selection, chromosomal integration and cassette amplification. The *cat*H gene is present on a larger native *B. licheniformis* chromosomal fragment surrounding the coding sequence with upstream and downstream sequences. Part of the upstream *cat*H sequence (called 5' repeat) is present twice on the plasmid to allow amplification of the expression cassette on the chromosome. The expression cassette (containing a native promoter and signal sequence and the KLM3´ gene) is located between these repeats.

The only part of the integration vector that is left in the final *B. licheniformis* production strain is the integration cassette, consisting of the synthetic modified KLM3' GCAT enzyme encoding gene of the donor strain *Aeromonas salmonicida* subsp. *salmonicida* gene plus the endogenous *B. licheniformis* chloramphenicol acetyl transferase gene including a duplicated 5' sequence. This integration cassette is integrated in the chromosome of the production strain, at the site of the endogenous (deleted) chloramphenicol resistance gene. There are no plasmid vector sequences left in the final production organism.

The genetic construction was evaluated at every step to assess the incorporation of the desired functional genetic information and the final construct was verified by Southern blot analysis to confirm that only the intended genetic modifications to the *B. licheniformis* strain had been made.

3 Pathogenicity and toxicity of the modified micro-organism

3.1 The production strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Pariza and Foster, 1983). If the organism is non-toxigenic and non-pathogenic, then it is assumed that foods or food ingredients produced from the organism, using current Good Manufacturing Practices, are safe to consume (IFBC, 1990). Pariza and Foster (1983) define a non-toxigenic organism as 'one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure' and a non-pathogenic organism as 'one that is very unlikely to produce disease under ordinary circumstances.' *B. licheniformis* meets these criteria for non-toxigenicity and non-pathogenicity.

3.2 The donor

Aeromonas salmonicida subsp. *salmonicida* is considered a Class 2 organism in the EU (see e.g. the DSMZ website) but is Biosafety level 1 in the USA (see ATCC website).

Aeromonas salmonicida is a particularly virulent pathogen of salmonid fish. A secreted KLM3´ has in the past been considered as key virulence determinant of this bacterium (Lee and Ellis, 1990), like the KLM3´ of *Legionella pneumophila* was suggested to be involved in *Legionella* pathogenicity (Banerji et al, 2005). However, recent research using mutant strains has shown that KLM3´ is not essential for intracellular infection of *L. pneumophila* (Flieger et al, 2002). According to the WHO, *A. salmonicida* is a fish pathogen, but has not been associated with human infection (Sartory, 1998).

There are several published works examining the toxicity of KLM3´ from *Aeromonas salmonicida* on erythrocytes from both fish and mammalian sources (Lee and Ellis, 1990; Vipond *et al*., 1998; and Buckley *et a*l., 1982). Buckley et al. (1982) demonstrated that while the individual glycerophospholipids found in human erythrocytes could serve as substrates for KLM3´ *in vitro*, there was no difference in the hemolysis rate between erythrocytes treated with KLM3´ and controls after 60 minutes. The enzyme did not penetrate the bilayer but acted only on one side of the membrane. The authors concluded that virtually all of the

fatty acid hydrolyzed from the phospholipids was transferred to cholesterol in the membrane.

Vipond et al., (1998) states that proteolytic activation of pro-KLM3´ results in lysis of fish erythrocytes although not directly of mammalian erythrocytes and cites 2 additional references supporting this statement (Eggset et al., 1994; Ellis, 1997).

Lee and Ellis, (1990) provide a rationale for the specificity of KLM3´ for fish over mammalian erythrocytes. The preferred substrate for KLM3´ is phosphatidylcholine substituted with unsaturated fatty acids (Buckley, 1982) and fish tissues have higher contents of polyunsaturated fatty acids than those of mammals. For example, the erythrocyte membranes of Atlantic salmon contain 58.6% phosphatidylcholine while it comprises only 29.5% of human erythrocyte membranes.

Therefore the conclusion from the literature is that the hemolytic effect of KLM3´ is observed mainly in *in vitro* assays and the intact mammalian erythrocytes are not lysed. Further, the hemolytic effect of KLM3´ is highly specific. Fish erythrocytes on the other hand, are highly susceptible to lysis by KLM3´ due to a much higher percentage of phosphatidylcholine (2x) than that found in mammalian erythrocytes. This evidence strongly suggests that KLM3´ is not a hemolysin for mammalian erythrocytes.

3.3 The host

Bacillus licheniformis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide.

Mixed carbohydrase and protease from *Bacillus licheniformis* were affirmed as GRAS by FDA on January 4, 1983 (48 FR 239). Also the FDA has no questions to five GRAS notices on enzymes derived from *Bacillus licheniformis*:

- Alpha-amylase derived from *Bacillus licheniformis* carrying a gene encoding a modified alpha-amylase derived from *Bacillus lichenformis* and *Bacillus amyloliquefaciens* (GRN No. 22),
- Alpha-Amylase derived from *Bacillus licheniformis* carrying a gene encoding alpha-amylase from *Bacillus stearothermophilus* (GRN No. 24),
- Pullulanase derived from *Bacillus licheniformis* carrying a gene encoding pullulanase from *B. deramificans* (GRN No. 72),
- Alpha-amylase derived from *Bacillus licheniformis* carrying a gene encoding a modified alpha-amylase from *Bacillus licheniformis* (GRN No. 79), and
- Glycerophospholipid cholesterol acyltransferase (KLM3´) enzyme preparation produced by *Bacillus licheniformis* expressing the gene

encoding KLM3´ enzyme from *Aeromonas salmonicida* subsp. *salmonicida* (GRN No. 265).

Amylase from *Bacillus licheniformis* has been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set (Technical Report Series 733, 1986).

Bacillus licheniformis, including genetically modified strains, has been approved for the production of amylase, protease and pullulanase enzymes in the food industry in Brazil (Resolução Da Diretoria Colegiada - RDC Nº 205, De 14 De Novembro De 2006).

Bacillus licheniformis, including genetically modified strains, have been approved for the production of amylase enzymes in the food industry in France and it is also approved for the production of alpha-amylase, proteases, cyclomaltodextrin transferase and pullulanase (Arrêté du 19 Octobre 2006 as amended).

Strains of *B. licheniformis* are found in Table V of Division 16 of "Canadian Food and Drugs Act and Regulations", as an authorized source for amylases and proteases in several food applications.

The species *Bacillus licheniformis* is an accepted source of safe food enzymes in the literature. The safety of *B. licheniformis* strains was recently reviewed (Priest, et al, 1994).

Pathogenic strains of *Bacillus licheniformis* are NOT described in the Bergey Manual or in the ATCC and other catalogues. The species *Bacillus licheniformis* does not appear on the Proposal for a Council Directive amending the "Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work".

Strains of *B. licheniformis* are found in the Sixth edition of "Food Chemicals Codex" as a source for the enzymatic preparation of carbohydrase and protease used in the treatment of foods.

The parent strain of the current production strain and its progeny, *B. licheniformis* BRA7 & characterized derivatives, have been used for the production of α-amylase enzyme preparations since 1989, as well as for the production of proteases, pullulanase and xylanase by Genencor.

Numerous feeding, mutagenicity and carcinogenicity studies using enzyme products from *B. licheniforms* Bra7-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

3.4 Allergenicity of KLM3´

As a protein, enzymes have the potential to cause allergic responses. Although virtually all allergens are proteins, it is noteworthy that only a small percentage of all dietary proteins are food allergens. Below we describe briefly why ingestion of enzymes used as food processing aids is unlikely to elicit an allergic response after consumption.

Enzymes are proteins with highly specialized catalytic function. They are produced by all living organisms and are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings. Enzymes are essential for all metabolic processes and they have the unique ability to facilitate biochemical reactions without undergoing change themselves. As such, enzymes are natural protein molecules that act as very efficient catalysts of biochemical reactions. Like many other proteins, enzymes may have the potential to cause allergic responses, primarily after inhalation exposure. According to Pariza and Foster (1983) allergies represent only a very minor food safety concern in regard to food processing enzymes. Allergic reactions after consuming enzymes could happen, but are scarce (Dauvrin et al, 1998). Poulsen (2004) reported that ingestion of an enzyme does not commonly result in the corresponding food allergy in individuals with inhalation allergy to a particular enzyme. Bindslev-Jensen et al (2006) conducted extensive studies in individuals with allergies with a variety of ingested food enzymes (carbohydrases, lipases, proteases) and confirmed that they are not food allergens, regardless of microbial source (bacterial or fungal) or the techniques used to produce them, including rDNA modification and protein engineering. These and other reports allow us to conclude that ingestion of food enzymes is not considered to be a concern with regards to food allergy. This may due to difference in exposure pattern (digestive route vs. inhalation route), insignificant exposure level in final foods, inactivation through processing, or molecular structure.

Despite this lack of general concern, the potential that KLM3´ could be a food allergen was examined (for details, see Appendix B2). The full KLM3´ sequence was compared with known allergens using either the SDAP database (containing 737 allergens or the Allermatch database combined database containing 792 allergens. KLM3´ does not match with any allergens, using 0.01 as the maximum score to indicate homology.

A secondary, more detailed search for exact matches of short stretches (6 amino acids) of sequence that could serve as potential IgE binding sites established the existence of 1 such 6-amino acid stretch (LAPTGM) also present in profilin allergens in timothy grass pollen (Asturias et al. 1997; Marknell DeWitt et al, 2002; Valenta et al, 1994), sunflower pollen (Asturias et al, 1998), and mugwort pollen (Wopfner et al, 2002), but not in any food allergens.

Further protein hydrophobicity analysis indicated this 6-amino acid stretch in the KLM3´ sequence not to be hydrophilic and hence not likely exposed to the surface of a folded protein, thus not likely presenting an antigenic epitope.

Furthermore, no IgE epitopes are described for the profilin hits in the SDAP database: Phl, Ph1, and Hel a 2 (the Mugwort profilin is not listed in SDAP).

Taking all the above into account, we conclude that KLM3´ does not match any known food allergens.

4 Genetic stability of the source organism

The production strain proved to be 100% stable after at least 60 generations of fermentation, judged by chloramphenicol resistance and KLM3´ production.

5 Pariza-Johnson Decision tree

Pariza and Johnson (2001) have published guidelines for the safety assessment of microbial enzyme preparations. These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations.

Danisco has evaluated KLM3´ according to the safety scheme of Pariza and Johnson (2001) (Appendix B3) and determined that this enzyme preparation is safe for use in food as a processing aid. This determination employed an extensive review of published and unpublished safety data available on the enzyme, the production organism, the enzyme manufacturing process, and the enzyme product (Pariza and Johnson, 2001)

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Appendix B1 : Certificate of analysis of test items

Genencor International®

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CERTIFICATE OF ANALYSIS

Name of Test Article: **ACYLTRANSFERASE BL1**

Production/Strain Name: *Bacillus licheniformis* **BML780-KLM3' CAP50.**

Production Site: Rochester, USA

Genencor International Culture Collection Number: GICC 3265

Designation of Lot Tested: 20068010

Description: Clear brown liquid

Expiration Date: Stable for at least 1 year from date of issuance when stored frozen

All of the analytical studies listed below were conducted in accordance with GLP regulations and ISO 9002 standards.

RESULTS:

1. Activity: 1156 U/ml
2. Total and TCA Protein
   The samples were measured for TCA and total protein by nitrogen analysis (with a KLM3' conversion factor of 5.96 g protein/g nitrogen).

Total Proteins:	30.40 mg/ml
TCA Proteins:	14.13 mg/ml
% Total Organic Solids: (100% – moisture% – ash%)	8.67%

3. Specific gravity: 1.021 g/ml
4. pH: 6.30
5. Inorganic materials

% Ash:	1.05%
% moisture:	90.28%

6. Microbial analysis: Microbial analysis conducted by GCOR, Rochester, NY

Analysis	Results
Total viable count	< 1CFU/ml
Coliform	< 1CFU/ml

E. Coli	negative/25 ml
Salmonella	negative/25 ml
Staphylococcus aureus	< 1 CFU/ml
Production strain	negative
Anaerobic sulfite reducers	negative
Antibiotic activity assay	negative

7. Mycotoxin analysis: Not applicable
8. Heavy metals analysis (conducted at Silliker Laboratories)

Analysis	Results
Heavy metals as Pb	< 30 ppm
Arsenic	< 3 ppm
Lead	< 0.5 ppm
Mercury	< 0.5 ppm
Cadmium	< 5 ppm

9. Stability Data :

Room Temperature (all activity units are reported in U/ml)

Sample ID	Dilution	T = 0	T = 5 hours	% of T = 0
20068010	straight	1094	1088	99.5
20068010	1/2	542	554	102.2
20068010	1/4	274	270	98.5

Refrigerator (4C): Undiluted Material

Sample ID	Dilution	*T=0*	*T = 7 days*	% of T = 0
20068010	straight	1094	851	77.8
20068010	1/2	542	441	81.3
20068010	1/4	274	220	80.2

Frozen (-20°C) : Undiluted Material

Sample ID	Dilution	T = 0	T = 30 days	% of T = 0	T = 60 days	% of T = 0	T = 90 days	% of T = 0
20068010	straight	1094	1097	100.3	1120	102.4	1106	101.1
20068010	1/2	542	557	102.8	551	101.7	529	97.6
20068010	1/4	274	271	98.9	277	101.1	229	83.6

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CERTIFICATE OF ANALYSIS

Name of Test Article: **ACYLTRANSFERASE BL1** .

Production/Strain Name: *Bacillus licheniformis* **BML780-KLM3' CAP50.**

Production Site: Rochester, USA

Genencor International Culture Collection Number: GICC 3265

Designation of Lot Tested: 20068010

Description: Lyophilized Powder

Analytical studies 3-5 listed below were conducted in accordance with GLP regulations and ISO 9002 standards.

RESULTS:

1. Activity: 21512 LATU/g

2. Dry Matter: 89%

3. Microbial analysis: Microbial analysis conducted on the liquid test material prior to lyophilization by GCOR, Rochester, NY

Analysis	Results
Total viable count	< 1CFU/ml
Coliform	< 1CFU/ml
E. Coli	negative/25 ml
Salmonella	negative/25 ml
Staphylococcus aureus	< 1 CFU/ml
Production strain	negative
Anaerobic sulfite reducers	negative
Antibiotic activity assay	negative

4. Mycotoxin analysis: Not applicable

5. Heavy metals analysis (conducted on the liquid test material prior to lyophilization at Silliker Laboratories)

Analysis	Results
Heavy metals as Pb	< 30 ppm
Arsenic	< 3 ppm
Lead	< 0.5 ppm
Mercury	< 0.5 ppm
Cadmium	< 5 ppm

6. Stability Data : Lyophilized powder is stable for at least 1 year when stored frozen

Bio-Analytical Representative:
Jorn Borch Soe Date: 25. August 2006
Jorn Borch Soe

Study Sponsor's Representative
Hanne Valsted Thygesen Date: 25 Aug 2006
Hanne Valsted Thygesen

Study Monitor's Representative:
Quang Q. Bui Date: Aug 28, 2006
Quang Q. Bui

Appendix B2: Risk assessment for potential food allergenicity

A general BLAST search using the mature KLM3´ protein sequence demonstrates significant homology (score <0.01) with over 300 protein sequences, mostly of other acyltransferases and lipolytic proteins. FASTA alignments of the full KLM3´ sequence with known allergens using either the SDAP database (http://fermi.utmb.edu/SDAP/sdap_who.html)containing 737 allergens or the Allermatch database combined database (www.allermatch.org) containing 792 allergens does not match KLM3´ with any allergens, using 0.01 as the maximum score to indicate homology.

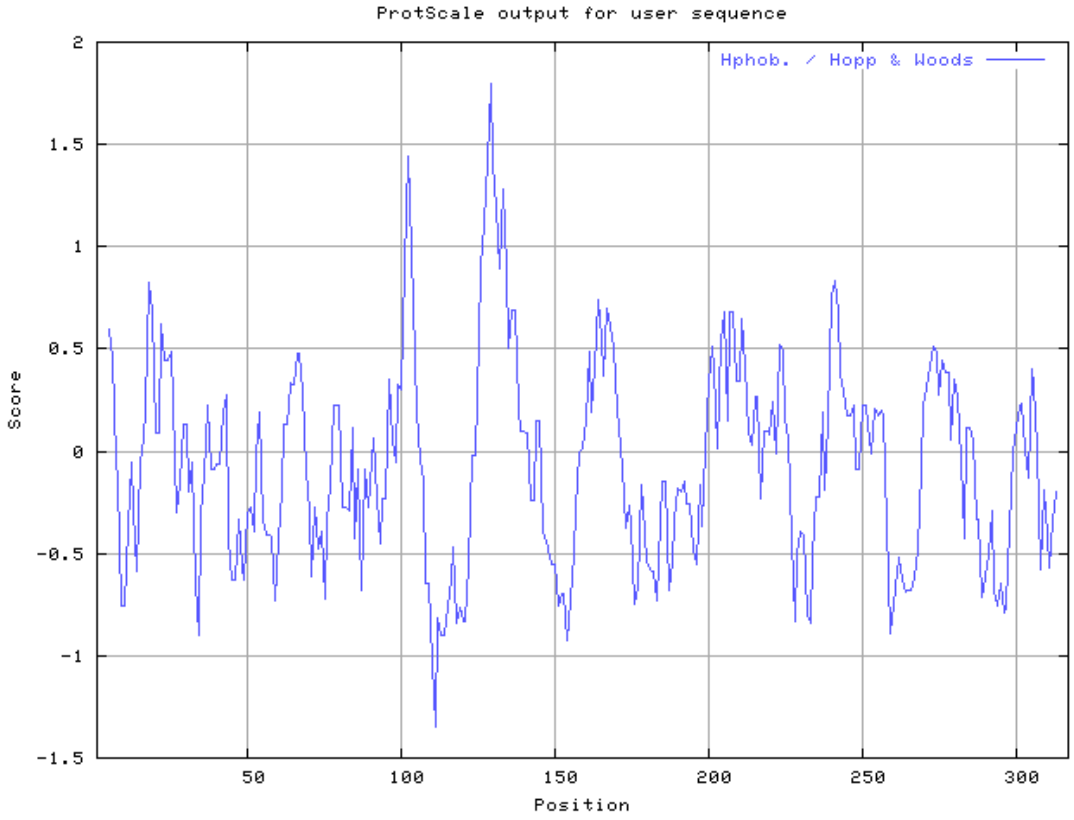
Following the recommendations in FAO/WHO (2001), two additional types of searches against the combined Allermatch database containing non-redundant allergen sequences from the SwissProt database and the WHO-IUIS list were performed using Allermatch software. The software algorithm and combined allergen database are also described in Fiers et al. (2004), http://www.biomedcentral.com/content/pdf/1471-2105-5-133.pdf.

The initial search according to the FAO/WHO guidelines, using a sliding window of 80-amino acid stretches and greater than 35% identity to indicate a match, revealed no matches of KLM3´ with known allergens. A secondary, more detailed search for exact matches of short stretches (6 amino acids) of sequence that could serve as potential IgE binding sites established the existence of 1 such 6-amino acid stretch (LAPTGM) also present in profilin allergens in timothy grass pollen (Asturias et al., 1997; Marknell DeWitt et al., 2002; Valenta et al., 1994), sunflower pollen (Asturias et al., 1998), and mugwort pollen (Wopfner et al., 2002), but no matches with any food allergens.

Further protein hydrophobicity analysis with ExPaSy ProtScale software (http://www.expasy.org/cgi-bin/protscale.pl) using the Hopp & Woods hydrophobicity algorithm (Hopp and Woods, 1981) indicated this 6-amino acid stretch in the KLM3´ sequence not to be hydrophilic (see Figure 1), and hence not likely exposed to the surface of a folded protein, thus not likely presenting an antigenic epitope. Furthermore, no IgE epitopes are described for the profilin hits in the SDAP database: Phl p 11 (http://fermi.utmb.edu/cgi-bin/SDAP/sdap_02?dB_Type=0&allid=554), Ph1 p 12 (http://fermi.utmb.edu/cgi-bin/SDAP/sdap_02?dB_Type=0&allid=35), and Hel a 2 (http://fermi.utmb.edu/cgi-bin/SDAP/sdap_02?dB_Type=0&allid=14) (the Mugwort profilin is not listed in SDAP).

We conclude that KLM3´ does not match any known food allergens.

Figure 1: Hydrophilicity profile of KLM3´ protein, using the Hopp & Woods (1981) hydrophobicity scores for individual amino acids window size of 9 (default value). The 6 amino acid stretch in question runs from position 191-196 and has scores below 0 which means it isn't hydrophilic.



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Valenta, R, T. Ball, S. Vrtala, M. Duchene, D. Kraft, O. Scheiner. 1994. cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. Biochem. Biophys. Res. Commun. 199:106-118.

Wopfner, N., M. Willeroider, D. Hebenstreit, R. van Ree, M. Aalbers, P. Briza, J. Thalhamer, C. Ebner, K. Richter, and F. Ferreira. 2002. Molecular and Immunological Characterization of Profilin from Mugwort Pollen. Biol. Chem. 383: 1779–1789.

Appendix B3 : Analysis of safety based on Pariza/Johnson Decision tree

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme preparations (2001). These guidelines are based upon decades of experience in the production, use and safety evaluation of enzyme preparations. The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that the Food Pro LysoMax Oil product is "ACCEPTED" as safe for its intended use.

1. **Is the production strain genetically modified? -** Yes, go to 2;

2. **Is the production strain modified using rDNA techniques? –** Yes, go to 3;

3. **Issues relating to the introduced DNA are addressed:**
    a. **Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food? –** No, this enzyme has not been used in food processing before. Go to 3b;
    b. **Is the NOAEL for the text article in appropriate short term oral studies sufficiently high to assure safety? –** Yes, the 872X safety factor in the 91-day study (with Ames and Mouse Micronucleus Studies negative) is high enough to assure safety. Go to 3c;
    c. Is the test article free of transferable antibiotic resistance gene DNA? – Yes, Go to 3e;
    e. Is all other introduced DNA well characterized and free of attributes that would render it un-safe for constructing microorganisms to be used to produce food-grade products? – Yes, Go to 4;

4. **Is the introduced DNA randomly integrated into the chromosome? –** No, the gene integrated at the *cat*H locus of the *B. licheniformis* genome; Go to 5;

5. **Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleitropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? –** Yes, Go to 6;

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? –** Yes, *B. licheniformis* safety as a production host and methods of modification are well documented and their safety have been confirmed through toxicology testing - Accept.

Conclusion: Article is accepted

June 28, 2010

APPENDIX C: Dietary exposure

Contents:

1 Application areas

KLM 3' will be used in egg yolk and whole eggs, in processed meats, in degumming of vegetable oils, in milk products, and in bakery products containing eggs like cake products. In all of these applications, KLM3' will be used as a processing aid where the enzyme is either not present in the final food or present in insignificant quantities having no function or technical effect in the final food.

Consequently, according to Standard 1.3.1 on food additives, the application areas are:

- Dairy products excluding butter and fats (category 1)
- Edible oils and fats (category 2)
- Bread and bakery products (category 7)
- Meat and meat products – processed meat (category 8.2)
- Egg and egg products (category 10)

2 Level of use

KLM3' will be added to eggs for mayonnaise production at a maximum of 5000 LATU/kg food; to processed meats at 300 LATU/kg food; to oil for degumming at a maximum of 1000 LATU/kg food; and to eggs used in bakery products at a maximum of 500 LATU/kg food.

KLM3' is proposed to be used in UHT (Ultra High Temperature) milk and milk dairy products at the following dosage levels:

- UHT milk: at 5-20 LATU/kg milk (corresponding to 0.1335 to 0.534 mg total protein/kg milk)
- Milk powder: at 5-20 LATU/kg milk used for milk powder (1 kg milk yields 9-15% milk powder)
- Yogurt: at 5-20 LATU/kg milk used for yogurt

3 Level of residues in food

3.1 Estimated Food Intake

- Use in degumming of oils

KLM1 can be used for degumming of oils to improve the quality and taste of the oil. The enzyme will be added to the oil in a water phase and will not migrate to the oil phase. After treatment of the oil the enzyme will be removed together with hydrolysis products during a washing process. Should any residues of the enzyme be present in the oil, they will be removed during the further refining of the edible oil.

As KLM1 is then not entering the food via oil, intake of oil should not used as basis for the calculation of intake of KLM1. However, to account for any residues present in edible oil, the intake of such products is estimated to be 50 g/person/day to illustrate a worst-case scenario.

- Use in egg products for baking purpose

In US, intake of eggs in baked goods is included in the intake data for grain products according to the USDA CSFII, 1994-1996. The mean intake of such products is 57 g/person/day (food subgroups quick breads, pancakes, French toast, cakes, cookies, pastries, pies). However, the major part of these foods is other ingredients than eggs. Danisco estimates that the content of eggs in these foods is no more than 10% resulting in an estimated intake of 6 g/person/day.

In Denmark, according to data from the Danish Food Survey,1995 (Andersen et al., 1996), the mean intake of eggs in DK is 17 g/person/day. This however includes egg, which are eaten fresh as well as eggs used in omelettes, cakes and other foods. Danisco estimates that no more than 10 g/person/day is used in cakes in Denmark.

- Use in mayonnaise

In US, intake of mayonnaise in the USA is included in the intake of salad dressings according to the USDA CSFII, 1994-1996. Mean intake of salad dressing is 8 g/person/day. However, the dose of the enzyme will be calculated based on the amount of egg present in mayonnaise. Typical egg content in mayonnaise is 4% and the intake of egg via mayonnaise is then 0.32 g/person/day.

In Denmark, intake of mayonnaise is included in the intake of fats and oils, which is 45 g/person/day as a mean value according to data from the Danish Food Survey,1995 (Andersen et al., 1996). However, the intake of fats used on bread is 20 g/person/day, which should be subtracted. The group also includes fats and oils used for frying and for remoulade. Danisco estimates this intake to be 15 g/person/day and the intake from mayonnaise is consequently estimated to be no more than 10 g/person/day. However, the dose of the enzyme will be calculated based on the amount of egg present in mayonnaise. Typical egg content in mayonnaise is 4% and the intake of egg via mayonnaise is then 0.4 g/person/day.

- Use in processed meat

The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007).

US Population	Food Intake gram/kg bw/day	Max Enzyme Dose – LATU/kg food	Max Enzyme/Day Units/kg bw/day	Max Total Protein/kg bw/day [1]
Oils	**0.83**	1000	0.83	
Eggs/bakery	0.10	500	0.05	
Eggs/mayonnaise	**0.02**	5000	0.10	
Processed meat	0.67	300	0.20	
TOTAL for US			**1.18**	**0.032**
Danish Population				
Oils	**0.83**	1000	0.83	
Eggs/bakery	**0.17**	500	0.09	
Eggs/mayonnaise	**0.02**	5000	0.10	
Processed meat	**0.70**	300	0.21	
TOTAL for DK			**1.23**	**0.033**

[1] 1 LATU ml = $\frac{30.40 \text{ mg total protein/ml}}{1142}$ = 0.0267 mg total protein/ml

- Use in milk products

The consumption of UHT milk varies with countries and Belgium and France as the highest (being 96.7 and 95.5% of total milk consumption) and Denmark as the lowest (0.1% of total milk consumption). Ultra high temperature processing applies to milk but is also used for cream and yogurt. In this risk characterization, the following defaults are used to represent a worst case scenario:

a. The consumption of milk and dairy based products from Belgium is used to calculate the daily human consumption (EFSA, 2008)
b. Milk powder and yogurt consumed in Belgium are derived from UHT milk
c. 100% of KLM'3 remain in the UHT milk and derived dairy products
d. Intake from "consumers only" is used since it represents a higher intake than the "general population"
e. The highest proposed dosage of 20 LATU/kg milk is used

Food Category - Belgium	**Mean**	**P95**	**P97.5**
Total milk and dairy based products (consumers only)	211 g/day	539 g/day	**689 g/day or 11.5 g/kg bw/day**
Daily intake of KLM 3' applied at 20 LATU/kg milk	4.22 LATU/day	10.78 LATU/day	13.78 LATU/day
Daily intake of KLM 3' total protein (1 LATU = 0.0267 mg total protein)	0.113 mg total protein/day	0.287 mg total protein/day	0.365 mg total protein/day

Daily intake of KLM 3' total protein per kg body weight (default = 60 kg person)	0.0018 mg total protein/kg bw	0.0047 mg total protein/kg bw	0.0061 mg total protein/kg bw

3.2 Estimated intake of KLM3´

As noted above, KLM3' is expected to be removed during the subsequent production processes for all applications.

During oil processing, the enzyme will go with the water phase, which together with the meal is toasted before use for animal feed and the heat will denature the enzyme. Any residual enzyme in the oil is removed and denatured during refining and deodorization.

After treatment of egg yolk, the yolks or food, mayonnaise and baked goods, are pasteurized or baked before consumption; the heat and pressure will denature the enzyme.

The enzyme is also inactivated during cooking of processed meat.

Heat treatment of the enzyme KLM3´during the normal UHT, powdered milk and yoghurt process causes a complete inactivation of the enzyme, and no activity is left in the final product.

FOOD COMMODITIES	Food Intake gram/kg bw/day	Max Enzyme Dose – LATU/kg food	Max Enzyme Dose - LATU/kg bw/day	Max Total Protein/kg bw/day (1)
Oils	0.83	1000	0.83	
Eggs/bakery	0.17	500	0.09	
Eggs/mayonnaise	0.02	5000	0.10	
Processed meat	0.70	300	0.21	
UHT milk and dairy products	11.5	20	0.23	
Cumulative			**1.46**	**0.039**

The maximum estimated daily intake of KLM3' from all dietary sources is 0.053 mg total protein/kg bw/day under the scenario that (1) all above commodities are treated with KLM3', (2) 100% of KLM3' remains in the product after processing, and (3) all consumers eat all these commodities treated with KLM3'. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing.

Maximum Estimated Daily Intake of KLM3' = 0.039 mg/kg bw

4 Safety assessment

Acyltransferase BL1 (KLM3') is an enzyme produced from *Bacillus licheniformis* strain BML 780-KLM3' CAP50 (GICC 3265) which was genetically modified to express the acyl transferase gene from *Aeromonas salmonicida* subsp. *salmonicida*.

To assess the safety of KLM3' in foods, different endpoints of toxicity were investigated at LAB Scantox (Denmark) and are evaluated and assessed in this document.

According to the Directive of the Commission 93/21/EEC of April 27, 1993, KLM3' is non hazardous based on acute oral studies. In genotoxicity studies, KLM3' is not mutagenic, clastogenic or aneugenic. Daily oral administration of KLM3' up to and including a dose level of 41 mg total protein/kg bw/day or 116.90 mg TOS/kg bw/day does not result in any manifestation of systemic, hematologic, or histopathologic adverse effects.

- ***Identification of the NOAEL***

In the 90-day oral (gavage) study in rats (Scantox No. 62129), a NOAEL was established at 41 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day). The study was conducted in compliance with both the FDA Good Laboratory Practice Regulations and the OECD Good Laboratory Practice and was designed based on OECD guideline No. 408. Since human exposure to KLM3' is through oral ingestion, selection of this NOAEL is thus appropriate.

No Observed Adverse Effect Level = 41 mg total protein/kg bw/day

Based on the results from the 90-day oral (gavage) feeding study cited above

$$\text{Margin of safety} = \frac{\text{No observed adverse effect level}}{\text{Maximum Estimated Daily exposure}}$$

Margin of safety = $\frac{41.0 \text{ mg/kg bw/day}}{0.039 \text{ mg/kg bw/day}}$ = 1051

5 Conclusion

The safety of Acyltransferase (KLM3') as a food processing aid in degumming of oil in egg yolk and whole eggs for cakes and mayonnaise, in processed meats and in UHT milk and dairy products is assessed in a battery of toxicology studies investigating its acute oral, mutagenic and systemic toxicity potential. KLM3' is not acutely toxic. KLM3' is not a mutagen, not a clastogen, not an aneugen and does not increase the formation of micronuclei in bone marrow erythrocytes. Although KLM3' demonstrates cytotoxicity in *in vitro* cultures, no similar adverse effects are found in the whole animal. Daily administration of KLM3' for 90 continuous days did not result in overt signs of systemic toxicity. A NOAEL is established at 41.0 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day).

Based on a worst-case scenario that a person is consuming KLM3' from the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise and in processed meat products (i.e., cumulative risk), this NOAEL still offers an 1051X fold margin of safety.

6 References

Danish Food Survey, Andersen, N.L., et al. 1996. Danskernes kostvarer 1995. Hovedresultater. Levnedsmiddel-styrelsen. Publikation nr. 235

Euromonitor international, national statistics, 2007

European Food Safety Authority (EFSA), March 2008, Data Collection and Exposure Unit (DATEX), Belgian National Food Consumption Survey, reference year: 2004

Scantox Study No. 62123, Acute oral toxicity study in the rat with KLM3', September 2006

Scantox Study No. 62126, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, KLM3', 2006.

Scantox Study No. 62127, Acyltransferase BL1, Ames Test, October 2006.

Scantox Study No. 62129, a 13-week oral (gavage) toxicity study in rats with Acyltransferase BL1 (KLM3'), October 2006.

Scantox Study No. 64415, Mouse micronucleus test with Acyltransferase BL1, November 2006.

USDA, Results from USDA's 1994-96 Continuing Survey of Food Intakes by Individuals and 1994-96 Diet and Health Knowledge Survey

June 28, 2010

APPENDIX D: International and other National Standards

Contents:

Appendices D

D1	GRAS notice
D2	GRAS letter
D3	EU legislation on enzymes

1 Codex Standards

1.1 The enzyme

KLM3´ has not been evaluated by the Joint Expert Committee on Food Additives(JECFA)

1.2 Supporting evaluations

Amylase from *Bacillus licheniformis* has been reviewed by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO and an acceptable daily intake (ADI) "not specified" has been set (Technical Report Series 733, 1986).

2 International Legislation

2.1 United States

2.1.1 The enzyme

KLM3´ has been determined to be GRAS as a food processing aid in degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products by a panel of scientific experts in the USA (Appendix D2). In May 2009, the panel was reconvened to review the additional uses of the enzyme in UHT and powdered milk, and facilitating improved fermentation and viscosity formation during yoghurt culturing. It is also the subject of GRAS Notice 265 with a concurrence letter received from FDA, dated June 13, 2009 (Appendix D1). In this submission, the use in UHT milk is not considered. With this substitution, the Maximum Estimated Daily Intake of KLM3´ is reduced from 0.047 mg/kg bw to 0.034 mg/kg bw and the margin of safety is greater than that determined in the GRAS assessment.

2.1.2 Supporting approvals

Mixed carbohydrase and protease from *Bacillus licheniformis* were affirmed as GRAS by FDA on January 4, 1983 (48 FR 239). Also the FDA has no questions to five GRAS notices on enzymes derived from *Bacillus licheniformis*:

- Alpha-amylase derived from *Bacillus licheniformis* carrying a gene encoding a modified alpha-amylase derived from *Bacillus lichenformis* and *Bacillus amyloliquefaciens* (GRN No. 22),
- Alpha-Amylase derived from *Bacillus licheniformis* carrying a gene encoding alpha-amylase from *Bacillus stearothermophilus* (GRN No. 24),
- Pullulanase derived from *Bacillus licheniformis* carrying a gene encoding pullulanase from *B. deramificans* (GRN No. 72),

- Alpha-amylase derived from *Bacillus licheniformis* carrying a gene encoding a modified alpha-amylase from *Bacillus licheniformis* (GRN No. 79), and
- Glycerophospholipid cholesterol acyltransferase (KLM3´) enzyme preparation produced by *Bacillus licheniformis* expressing the gene encoding KLM3´ enzyme from *Aeromonas salmonicida* subsp. *salmonicida* (GRN No. 265).

2.2 Europe

2.2.1 The enzyme

KLM3´ has neither been evaluated in France nor in Denmark yet.

In Europe, most of the enzyme preparations used in food processing are considered processing aids, meaning that they have their technological function in the food-processing stage and not in the final food. They are excluded from the Food Additives Framework Directive. On 16 December 2008 the European Parliament and the Council adopted Regulation 1332/2008 EC on food enzymes which aims to harmonise authorisation and safety assessment procedures of enzymes used in food processing in the EU (Appendix D3). Several years will be needed for the new rules to become fully applicable across the EU. Until then, all national provisions on the use of food enzymes in individual EU Member States remain valid and applicable. Only France, Denmark and the United Kingdom have legislation covering all food-use enzymes. In Denmark, approval is needed prior to use. The information contained in the application dossier necessary for approval should follow the guidelines laid down by the SCF in 1992. Approval is valid only for a specific brand or trade name. In The United Kingdom, the approval is voluntary but recommended. The Department of Health bases it approval of an enzyme on the basis of its need and safety. The safety is evaluated by the Committee on the Toxicity of Chemicals in Foods, Consumer Products and the Environment. In the other EU countries, enzyme preparation should be proved to be safe for use in food before being sold in EU according to the General EU Food Law.

2.2.2 Supporting approvals

Bacillus licheniformis, including genetically modified strains, has been approved for the production of amylase enzymes in the food industry in Denmark and in France. In France, it is also approved for the production of proteases, cyclomaltodextrin transferase and pullulanase (Arrêté du 19 Octobre 2006 as amended).

2.3 Other countries

2.3.1 The enzyme

KLM3´ is under review for approval in Brazil under the “Technical Regulation of Enzymes and Enzymes preparations to be used in food for human consumption". This Regulation lists the authorized enzymes with their origin (vegetal, animal or microorganism). Each enzyme should comply with their specifications. JECFA, FCC, FDA and IUB are used as references.

A dossier must be submitted to the ANVISA (National Surveillance Agency) for evaluation and addition to the positive list. The evaluation includes especially:

- A safety evaluation incl. toxicological information, production process, specifications
- Justification for use
- benefits of the enzyme use
- Identification of the enzyme (origin) and its specifications
- Estimated enzyme intake
- international approvals (used as references)

2.3.2 Supporting approvals

Bacillus licheniformis, including genetically modified strains, has been approved for the production of amylase, protease and pullulanase enzymes in the food industry in Brazil (Resolução Da Diretoria Colegiada - RDC Nº 205, De 14 De Novembro De 2006).

Strains of *B. licheniformis* are found in Table V of Division 16 of “Canadian Food and Drugs Act and Regulations”, as an authorized source for amylases and proteases in several food applications.

Appendix D1 : GRAS notice

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

June 13, 2009

Alice J. Caddow
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

Re: GRAS Notice No. GRN 000265

Dear Ms. Caddow:

The Food and Drug Administration (FDA) is responding to the notice, dated November 3, 2008, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on November 5, 2008, filed it on November 7, 2008, and designated it as GRAS Notice No. GRN 000265.

The subject of the notice is a glycerophospholipid cholesterol acyltransferase (GCAT) enzyme preparation from *Bacillus licheniformis* expressing a modified GCAT gene from *Aeromonas salmonicida* subsp. *salmonicida* (GCAT enzyme preparation). The notice informs FDA of the view of Genencor, a Danisco Division (Genencor), that the GCAT enzyme preparation is GRAS, through scientific procedures, for use as an enzyme to modify phospholipids to lyso-phospholipids and cholesterol ester in egg yolks to avoid product separation during mayonnaise production; processed meat products for emulsification, better consistency, and reduced cooking loss; cheese for increased yield; eggs to give a softer and more tender crumb in bakery products, and degumming of vegetable oils.

21 CFR 101.4 states that all ingredients must be declared by their common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. Our use of "GCAT enzyme preparation" in this letter should not be considered an endorsement or recommendation of that term as an appropriate common or usual name for the purpose of declaring the substance in the ingredient statement of foods that contain that ingredient. Issues associated with labeling and the appropriate common or usual name of a food are the responsibility of the Office of Nutrition, Labeling and Dietary Supplements in the Center for Food Safety and Applied Nutrition.

Commercial enzyme preparations that are used in food typically contain an enzyme component, which catalyzes the chemical reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the production organism and manufacturing process. In its notice, Genencor provides information about all the components of the GCAT enzyme preparation.

As part of its notice, Genencor includes the report of a panel of individuals (Genencor's GRAS panel) who evaluated the data and information that are the basis for Genencor's GRAS determination. Genencor considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Genencor's GRAS panel evaluated the safety of the production

organism and its lineage, the gene encoding the GCAT enzyme, the manufacturing process, intended uses in food processing, estimates of dietary exposure, unpublished studies, and relevant published literature. Based on this review, Genencor's GRAS panel concluded that the GCAT enzyme preparation is GRAS under the conditions of its intended use.

GCAT is an acyltransferase that catalyzes the transfer of acyl groups from phospholipids and glycolipids to sterols and other acceptor molecules. According to the classification of enzymes established by the International Union of Biochemistry and Molecular Biology, GCAT is identified by the Enzyme Commission number 2.3.1.43. Its accepted name is phosphatidylcholine-sterol *O*-acyltransferase and its systematic name is phosphatidylcholine sterol *O*-acyltransferase. The Chemical Abstract Service Registry number for this enzyme is 9031-14-5. Genencor notes that the amino acid sequence of the GCAT enzyme has been modified from that of the native GCAT enzyme from *A. salmonicida* subsp. *salmonicida* in only one amino acid, namely, an aspartic acid substitution for asparagine at position 80. Genencor states that this modification optimizes GCAT expression in *B. licheniformis*.

Genencor describes the host microorganism, *B. licheniformis* strain BRA7, as derived from a *B. licheniformis* strain lineage that has been used in the production of several commercial enzymes used in food processing including α-amylase, protease, pullulanase, and xylanase. Genencor states that *B. licheniformis* is nonpathogenic and nontoxigenic, meets the criteria for a safe production microorganism described by several expert groups, and is widely used by enzyme manufacturers for the production of enzyme preparations for use in human food.

Genencor describes the development of the host and production strains. The host strain BRA7 was modified by deletion of several enzyme activities (proteases and amylase), a sporulation gene, and the native chloramphenicol resistance gene (the *catH* gene). The strain was subsequently transformed with a plasmid vector carrying the expression cassette with the modified GCAT gene under the control of a *B. licheniformis* promoter and terminator and the native *B. licheniformis catH* gene. The *catH* gene was used for selection, chromosomal integration, and amplification of the expression cassette. After integration, all vector sequences were deleted. Genencor states that since the native *B. licheniformis catH* gene was used in the construction of the production strain, no new antibiotic resistance was introduced. Genencor also notes that the production strain was evaluated to confirm that it contains only the intended genetic modifications.

Genencor describes the manufacture of the GCAT enzyme using submerged fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis*. Each production batch is initiated from a lyophilized stock culture, which is tested for identity, absence of foreign microorganisms and enzyme generating ability before use. All fermentation steps are conducted under controlled conditions and monitored for microbial contamination. The GCAT enzyme is secreted to the fermentation broth and is subsequently recovered via several purification and concentration steps. The resulting liquid concentrate is stabilized with glycerol and potassium sorbate and tested to demonstrate that it meets the intended specifications. Genencor reports that the commercial GCAT enzyme preparation typically contains approximately 83 milligrams (mg) total organic solids (TOS) per gram (mg TOS/g) and that the enzyme preparation will be used according to the current good manufacturing practice at minimum levels necessary to achieve the intended technical effect.

Genencor states that enzyme products conform to the general and additional requirements for enzyme preparations as described in the Food Chemicals Codex (6th edition) and to the current (2006) General Specifications and Considerations for Enzyme Preparations Used in Food Processing established by the Joint FAO/WHO Expert Committee on Food Additives.

Genencor states that the GCAT enzyme preparation is intended for use in egg yolks in the production of mayonnaise, processed meat products, cheese, whole eggs in baked goods, and degumming of vegetable oils. The recommended use levels range from 22.6 to 376.9 milligrams total organic solids per kilogram of food (mg TOS/kg food). The enzyme is expected to be either removed and/or inactivated during the pasteurization or baking of products containing egg yolks or eggs, cooking of processed meat products, and the processing of vegetable oils. Genencor states that residual enzyme in cheese will be active unless the cheese is heated. Genencor estimates a maximum daily intake of the GCAT to be 0.047 mg of the enzyme protein per kg of body weight per day (mg/kg bw/d; equivalent to 0.13 mg TOS/kg bw/d) under the assumption that 100% of the enzyme added to egg yolks, eggs, processed meat products, cheese, and vegetable oils would remain in the final foods.

Genencor summarizes unpublished toxicological studies conducted with the GCAT enzyme which include acute and subchronic (13-week) oral toxicity study in rats, bacterial reverse mutation assay (Ames test), *in vitro* mammalian chromosomal aberration test, and *in vivo* mouse micronucleus test. Genencor states that the enzyme was not toxic in the acute toxicity study at the highest dose tested of 600 mg total protein per kg, and that no systemic toxicity was observed in the subchronic study at the highest dose tested of 117 mg TOS/kg bw/d. Genencor also concludes that the genotoxicity studies demonstrated no mutagenic, clastogenic, or aneugenic effects.

Genencor provides general information regarding the potential allergenicity of enzymes used in food processing. Genencor states that exposure to enzymes is very low, and even if they were potentially allergenic when ingested, the likelihood of allergic sensitization of consumers to these proteins is virtually nil. In addition, Genencor compares the amino acid sequence of GCAT to the sequences of known allergens contained in two allergen databases and concludes that the enzyme does not match the amino acid sequences of any known food allergens when tested according to the FAO/WHO (2001) recommendations.

Standards of Identity

In the notice, Genencor states its intention to use GCAT enzyme preparation in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Use in Meat, Poultry, and Egg Products

During its evaluation of GRN 000265, FDA consulted with the Risk and Innovations Management Division (RIMD), Office of Policy, Program, and Development (OPPD), of the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture. Under the Federal Meat Inspection Act, the Poultry Products Inspection Act, and the Egg Products Inspection Act, FSIS is responsible for determining

the efficacy and suitability of food ingredients in meat, poultry, and egg products as well as prescribing safe conditions of use. Suitability relates to the effectiveness of the ingredient in performing the intended purpose of use and the assurance that the conditions of use will not result in an adulterated product, or one that misleads consumers.

Regarding suitability, FSIS has no objection to the use of GCAT enzyme preparation at a level not to exceed 376.9 mg TOS/kg of total product formulation in products produced under the authority of the Egg Products Inspection Act, including egg yolks, dried egg yolks, and frozen egg yolks. FSIS notes that distributors of the enzyme preparation should notify processors of egg products of the need to review the activation process in order to ensure that a safe food is produced if that egg product is incubated at elevated temperatures for a period of time in order to activate the enzymes. This resulting product is amenable and therefore must be identified and declared on the label as an "egg product" directly followed by the ingredients statement.[1]

In addition, FSIS has no objection to the use of GCAT enzyme preparation, at a level not to exceed 22.6 mg TOS/kg of total product formulation, as an emulsifier in comminuted meat products.[2]

The enzyme will need to be listed by its appropriate common or usual name in the ingredients statement on the label of the products in which it is used as an ingredient. Also, the product formula, and their percentages in total, along with a product label, will need to be submitted to the FSIS, Office of Policy and Program Development, Labeling and Program Delivery Division, so that the label can be reviewed and evaluated.

Further questions regarding use in meat, poultry, and egg products should be directed to Dr. John M. Hicks, Jr., RIMD/OPPD/FSIS, via email at john.hicks@fsis.usda.gov or to Dr. Catherine Rockwell, RIMD/OPPD/FSIS, via email at catherine.rockwell@fsis.usda.gov.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FFDCA)

Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Genencor's notice that the GCAT enzyme preparation is GRAS for use as an enzyme to modify phospholipids to lyso-phospholipids and cholesterol ester in egg yolks to avoid product separation during mayonnaise production; processed meat products for emulsification, better consistency, and reduced cooking loss; cheese for increased yield; eggs to give a softer and more tender crumb in bakery products, and degumming of vegetable oils, FDA did not consider

[1] According to FSIS, the term amenable means "accountable or liable to an order or regulation."

[2] FSIS notes that comminuted meat products are a subset of processed meat products in which the sizes of meat pieces have been reduced by chopping, flaking, grinding, or mincing, but not by chunking or sectioning. FSIS's use of the term "comminuted" is based on technical effect data that the notifier submitted for review by FSIS. The data describe the effects of GCAT enzyme preparation on liver sausage, which is a comminuted meat product.

whether section 301(ll) or any of its exemptions apply to foods containing the GCAT enzyme preparation. Accordingly, this response should not be construed to be a statement that foods that contain the GCAT enzyme preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Genencor, as well as other information available to FDA, the agency has no questions at this time regarding Genencor's conclusion that the GCAT enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of the GCAT enzyme preparation. As always, it is the continuing responsibility of Genencor to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000265, as well as a copy of the information in this notice that conforms to the information in the proposed GRAS exemption claim (proposed 21 CFR 170.36(c) (1)), is available for public review and copying via the FDA home page at http://www.fda.gov. To view or obtain an electronic copy, follow the hyperlinks from the "Food" topic to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Sincerely,

Laura M. Tarantino, Ph.D.
Director,
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

cc: John M. Hicks, Jr. DVM, MPH
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Office of Policy and Program Development
Food Safety and Inspection Service
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Appendix D2 : GRAS panel letter

Expert Panel Report on the Safety and GRAS Status of the Proposed Uses in Food Processing of a GM-derived Glycerophospholipid Cholesterol Acyltransferase Enzyme (KLM3')
20 July, 2009
Modified 14 April, 2010

Introduction

A glycerophospholipid cholesterol acyltransferase (GCAT) enzyme produced by fermentation of a genetically modified (GM) strain of *Bacillus licheniformis* has been developed for use in food processing. This enzyme is designated as KLM3'.

Danisco USA convened a panel of expert scientists (the Expert Panel) qualified by scientific and biomedical training and national and international experience to evaluate the safety of foods and food ingredients to assess the safety and the GRAS status of the proposed uses of KLM3'. The resumes of the Panel appear in Section VI of the complete dossier. The panel was first convened at the end of 2007 to review the uses of this enzyme in egg yolk to be used in mayonnaise and baked goods, in processed meats, in degumming of oil and in milk products such as cheese. In May of 2009, the panel was reconvened to review the additional uses of the enzyme in UHT and powdered milk, and facilitating improved fermentation and viscosity formation during yogurt culturing.

Commercial enzyme preparations that are used in food processing typically contain an enzyme component which catalyzes the reaction that is responsible for its technical effect, as well as substances used as stabilizers, preservatives or diluents. Enzyme preparations may also contain constituents derived from the manufacturing process, e.g., components of the fermentation media or the residues of processing aids.

Danisco USA provided the Expert Panel with a dossier ("Expert Panel Report of the Safety and GRAS Status of the Proposed Uses in Food Processing of a GM-derived Glycerophospholipid Cholesterol Acyltransferease Enzyme KLM3'") containing summaries of published and unpublished information on KLM3' and other information deemed appropriate including:

- A detailed description of the GM microorganism and the glycerophospholipid cholesterol acyltransferase gene and enzyme;
- A discussion of the proposed uses in food processing;
- A safety evaluation including the safety of the production organism and its lineage; manufacturing process, dietary exposure data and reports of safety studies conducted on the enzyme;
- A discussion of the relevant safety literature; and
- An evaluation of the enzyme product according to the Pariza/Johnson decision tree (Pariza, M.W. and Johnson, E., 2001).

The Expert Panel independently critically evaluated these documents and other materials deemed relevant and conferred by telephone. The Expert Panel also conferred by telephone with representatives of Danisco USA and the Genencor Division of Danisco USA on December 4, 2007 and May 28, 2009, and considered all available information. The Expert Panel then met in executive session and developed the conclusion presented below.

Description, use and production

Glycerophospholipid cholesterol acyltransferase KLM3' is an enzyme that transfers acyl groups from phospholipids and glycolipids to acceptors such as sterols, fatty alcohols and other smaller primary alcohols. KLM3' will be used as follows:

- To modify phospholipid to lysophospholipid and cholesterol ester in egg yolk, improving its emulsification properties and avoiding separation during pasteurization when the modified egg yolk is used in mayonnaise and bakery products;
- To convert meat phospholipids to lysophospholipids, improving consistency and reducing cooking loss by improved emulsification of the fat in the meat;
- To convert phospholipids to lysophospholipids which are more water-soluble and can be removed from the oil by washing with water during oil degumming to improve the quality of the oil and prevent sedimentation in the oil;
- To convert milk phospholipids to lysophospholipids, improving emulsification and increasing cheese yield by entrapping more lipid in the cheese curd; and
- To transfer fatty acid acyl groups from milk phospholipid (or lecithin) to cholesterol resulting in the formation of lysophospholipids (or lysolecithins) and cholesterol esters, facilitating the formation of micelles and thereby reducing the build up of denatured proteins on heated surfaces during the production of UHT and powdered milk, and facilitating improved fermentation and viscosity formation during yogurt culturing.

Glycerophospholipid cholesterol acyltransferase KLM3' is a variant of the wild type glycerophospho-lipid cholesterol acyltransferase (GCAT) from *Aeromonas salmonicida* subsp. *Salmonicida* strain ATCC # 14174 and is expressed in a *B. licheniformis* strain constructed by Genencor. This *B. licheniformis* strain lineage has been used by Genencor as a host for the commercial production of a number of α-amylases for the starch liquefaction business since 1989, as well as for production of protease, pullulanase and xylanase. The gene, the vector and the host organism were fully characterized. The construction of the vector was documented in detail and the genetic stability of the recombinant organism was assessed and judged to be appropriate for the production of food grade materials.

Glycerophospholipid cholesterol acyltransferase KLM3'is secreted into the medium during aerobic fermentation and the enzyme is recovered by centrifugation and filtration and concentration via ultra-filtration.

Estimated exposure

The use of KLM3' in all of its intended applications results in a maximum consumption level (90^{th} percentile users) of 0.053 mg total protein /kg bw/day.

The estimated intake of Glycerophospholipid cholesterol acyltransferase KLM3' from all egg-products, oils, and cheese was calculated based on data from the USDA Continuing Survey of Food Intake by Individuals (USDA CSFII, 1997) and data from the Danish Food Survey in 1995 (Andersen et al., 1996). The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007). The estimated daily intake of milk and dairy based products from Belgium is used to calculate the daily human consumption (EFSA, 2008).

Safety

In assessing the safety of the production organism, Danisco relied on scientific review articles in support of its view that the safety of the production organism is the prime consideration in assessing the safety of an enzyme preparation intended for food use. Danisco reported that the host organism has a long history of safe industrial use and concluded that an enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nonpathogenic and nontoxigenic; the genetic information that is introduced into the host microorganism is well characterized; and the added DNA does not encode and express any known harmful or toxic substances. Danisco provided information to support the conclusion that the components of the enzyme preparation meet these standards and that the enzyme is produced using a safe strain lineage. The term *safe strain lineage* is defined and discussed in the Pariza-Johnson paper, as follows: "Thoroughly characterized nonpathogenic, nontoxigenic microbial strains, particularly those with a history of safe use in food enzyme manufacture, are logical candidates for generating a *safe strain lineage*, through which improved strains may be derived via genetic modification by using either traditional/classical or rDNA strain improvement strategies. The elements needed to establish a safe strain lineage include thoroughly characterizing the host organism, determining the safety of all new DNA that has been introduced into the host organism, and ensuring that the procedure(s) that have been used to modify the host organism are appropriate for food use."

Danisco provided unpublished toxicological studies performed on the KLM3' enzyme. These studies include:

Scantox Study No. 62125, Acute dermal irritation study in the rabbit with KLM3', September 2006;

Scantox Study No. 62124, Acute eye irritation/corrosion study in the rabbit with KLM3', September 2006;

Scantox Study No. 62123, Acute oral toxicity study in the rat with KLM3', September 2006;

Scantox Study No. 62127, Acyltransferase BL1, Ames Test, October 2006;

Scantox Study No. 62126, *In vitro* mammalian chromosome aberration test performed with human lymphocytes, KLM3', 2006;

Scantox Study No. 64415, Mouse micronucleus test with Acyltransferase BL1, November 2006; and

Scantox Study No. 62129, a 13-week oral (gavage) toxicity study in rats with Acyltransferase BL1 (KLM3'), October 2006.

The NOAEL in the 13-week gavage study in rats is 41.0 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day), the highest dose tested.

Reports of these studies are available from Danisco's Regulatory Affairs department in Palo Alto, CA. Additionally, a summary of the toxicology results has been published (S Evert, Q Bui, HV Thygesen, CN Edwards and M Jochumsen: Safety evaluation of Acyltransferase BL1 from *Bacillus licheniformis*Strain BML780-KLM3' CAP50 (GICC 3265) intended for food uses. International J of Toxicolgoy, Vol 28(1), P34, Jan/Feb 2009). Danisco concludes that the results of the toxicity and mutagenicity tests demonstrate the safety of the Danisco's glycerophospholipid cholesterol acyltransferase KLM3' preparation and support the safe use of enzyme preparations produced by the production strain.

It was also noted that:

- *Bacillus licheniformis* is well documented in the literature and through Genencor's experience as a safe host for enzyme production;
- KLM3' was evaluated via the Pariza-Johnson decision tree for safety of microbial enzymes and was "Accepted" as safe for its intended use; and
- The enzyme will be removed or inactivated during the subsequent production processes for all but the cheese application. During cheese production the enzyme will end up in the whey. The whey is often used for production of whey proteins and the enzyme will still be active if the food to which the whey proteins are added is not cooked. Residual enzyme in the cheese will also be active unless the cheese is heated.

Conclusion

We, the Expert Panel independently and collectively critically evaluated the data and information summarized in a dossier prepared by Danisco USA and subsequent documentation provided by Danisco USA and summarized above and conclude that the proposed uses in food processing of Glycerophospholipid cholesterol acytransferase KLM3', meeting appropriate food grade specifications described herein and manufactured consistent with current Good Manufacturing Practice, (cGMP) are safe.

We further conclude that the proposed uses of Glycerophospholipid cholesterol acytransferase KLM3' are Generally Recognized As Safe (GRAS) based on scientific procedures.

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

Herbert Blumenthal April 23, 2010

Herbert Blumenthal, PhD Date
Consultant

Joseph F. Borzelleca 21 April 2010

Joseph F. Borzelleca, PhD Date
Professor Emeritus of Pharmacology and Toxicology
Virginia Commonwealth University School of Medicine

Michael W. Pariza 26 April 2010

Michael W. Pariza, PhD Date
Professor of Food Science
Member, Michael W. Pariza Consulting LLC

Appendix D3 : EU Legislation on enzymes

Amfep/09/01 **Association of Manufacturers and Formulators of Enzyme Products** 16 January 2009

Amfep Statement on the EC Regulation 1332/2008 on Food Enzymes

On 16 December 2008 the European Parliament and the Council adopted Regulation 1332/2008 EC on food enzymes (OJ EU L 354/7, 31.12.2008).

The Association of Manufacturers and Formulators of Enzyme Products (Amfep) welcomes this EU legislation which is the first attempt ever to harmonise authorisation and safety assessment procedures of enzymes used in food processing in the EU. The Regulation is set to improve the functioning of the internal market by removing disparities among member states and bringing more legal certainty to the market.

Only authorised food enzymes will be allowed to be commercialised and/or used in foods sold in the EU – irrespective whether they are used as processing aids or ingredients. This also applies to imported foods. The European Food Safety Authority (EFSA) will play a pivotal role in the authorisation process of food enzymes. On the basis of EFSA's scientific advice, the EU Commission will grant authorisations after consulting member states and the EU Parliament.

The publication of the Regulation in the Official Journal of the European Union only marks the beginning of an extensive implementation process. In fact, several years will be needed for the new rules to become fully applicable across the EU. Until then, all national provisions on the use of food enzymes in individual EU Member States remain valid and applicable.

The European Commission, supported by EFSA, has until 2011 to specify what information is required to be submitted for a risk assessment of food enzymes. After that, the industry will have another two years (until 2013) to submit dossiers for evaluation and authorization of food enzymes presently used in food on the EU market. Only after the EU Commission and EFSA have completed the evaluation of all these dossiers will the first EU (positive) list of approved food enzymes be established. The Regulation 1332/2008 EC does not set a deadline by which this evaluation is to be completed.

Apart from the authorization requirements, Regulation 1332/2008 also lays down specific provisions on labelling of food enzymes, food enzyme preparations and food prepared with enzymes. The provisions on labelling of food enzymes and food enzyme preparations will enter into force on 20 January 2010, whereas the provisions on labelling of food prepared with enzymes enter into force on 20 January 2009. The latter do not increase the scope of the previous food labelling provisions, although some changes are introduced to the way the small number of food enzymes that are not used as processing aids are declared.

Amfep is working closely with relevant European Stakeholders to ensure a seamless implementation of the new EU legislation for the benefit of food enzyme manufacturers, their clients, and consumers.

NB: The proposal for a regulation on food enzymes is a part of a so-called Food Improvement Agents package (FIAP). While harmonising EU legislation for food enzymes, FIAP is also aiming at upgrading existing EU legislation on food additives (EC Regulation 1333/2008) and food flavourings (EC Regulation 1334/2008) and establishing a transversal authorisation procedure (EC Regulation 1331/2008) The EU Regulation on food additives will include a positive list of additives and carriers that will be allowed in food enzymes and food enzyme preparations. This list will come into force on 1 January 2011.

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