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A Danisco Division

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November 3, 2008

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Dr. Laura Tarantino, Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Tarantino:

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Genencor, a Danisco Division, ("Danisco") has determined that its glycerophospholipid cholesterol acyltransferase (GCAT) enzyme preparation produced by *Bacillus licheniformis* expressing the gene encoding GCAT enzyme from *Aeromonas salmonicida* subsp. *salmonicida* is a Generally Recognized as Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. We are hereby submitting, in triplicate, a Generally Recognized As Safe ("GRAS") Notification, in accordance with proposed 21 C.F.R. § 170.36, informing FDA of the view of Danisco that the GCAT enzyme preparation is GRAS, through scientific procedures, for use as an enzyme 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;
- Milk products like cheese to increase yield; and
- Bakery products containing eggs to give a softer and more tender crumb.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

GENENCOR

Glycerophospholipid Cholesterol Acyltransferase (GCAT) Enzyme Preparation from *Bacillus licheniformis* Expressing the GCAT Gene from *Aeromonas salmonicida subsp. salmonicida* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

November 3, 2008

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GENENCOR

1. GENERAL INTRODUCTION

FoodPro™ LysoMax® Oil is the Danisco trade name used for the GCAT enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding GCAT from *Aeromonas salmonicida subsp. salmonicida*, also called KLM3'. DNA encoding the GCAT protein was synthesized according to the known DNA sequence, modified at one amino acid position, and codon optimized for expression in *B. licheniformis,* and inserted into *B. licheniformis.*

The enzyme is intended 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; to improve the emulsification of processed meat products and contribute to better consistency and reduced cooking loss; for the degumming of vegetable oils; to increase yield in milk products like cheese; and to give a softer and more tender crumb in bakery products containing eggs.

The enzyme is a glycerophospholipid cholesterol acyltransferase (GCAT) (IUBMB 2.3.1.43) which catalyzes phosphatidylcholine + a sterol to 1-acylglycerophosphocholine + a sterol ester.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its GCAT enzyme preparation from a modified strain of *Bacillus licheniformis* expressing the GCAT gene from *Aeromonas salmonicida subsp. salmonicida* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of a GCAT enzyme preparation produced by a *B. licheniformis* host, which has been modified to express a gene encoding GCAT from *Aeromonas salmonicida subsp. salmonicida.*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for food use.[1,2] The safety of the production organism for this GCAT, *B. licheniformis,* is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material.[3-8] The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

GENENCOR

1.1 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.2 Common or Usual Name of Substance

GCAT enzyme preparation from *B. licheniformis* expressing the gene encoding GCAT enzyme from *Aeromonas salmonicida* subsp. *salmonicida.*

1.3 Applicable Conditions of Use

The GCAT enzyme is intended 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; to improve the emulsification of processed meat products and contribute to better consistency and reduced cooking loss; for the degumming of vegetable oils; to increase yield in milk products like cheese; and to give a softer and more tender crumb in bakery products containing eggs. The enzyme preparation is used at minimum levels to achieve the desired effect and according to requirements of normal production following current Good Manufacturing Practices. It is expected that mayonnaise, processed meats, cheese, degummed oils and bakery products produced using this GCAT will be consumed by the general population.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 Availability of Information for FDA Review

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism of the GCAT enzyme preparation is *B. licheniformis* strain GICC03265. It is derived by recombinant DNA methods from a strain of *Bacillus licheniformis* modified to express a synthetic GCAT gene DNA that has been modified at one amino acid and codon optimized for expression in *B. licheniformis* (see Section 2.2). *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[1] and several expert groups.[2-8] It contains the synthetic GCAT gene under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene.

The inserted DNA was integrated into the host on a in a vector derived from *Bacillus* plasmids pUB110 and pE194. The introduced DNA integrated into the Bacillus chromosome at the *cat* locus by Campbell type recombination. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences.

2.2 Host Microorganism

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 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 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 GCAT Expression 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 GCAT gene) is located between these repeats.

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.

2.4 Stability of the Introduced Genetic Sequences

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

2.5 Antibiotic Resistance Gene

The construction of the production organism utilized the native chloramphenicol resistance (CAT) gene from *B. licheniformis*; no new antibiotic resistance was introduced.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the IFBC[3] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Classification	Acyltransferase
IUB Nomenclature	phosphatidylcholine sterol O-acyltransferase
IUB Number:	2.3.1.43
CAS Number:	9031-14-5
EINECS Number:	232-796-2
Reaction catalyzed	phosphatidylcholine + a sterol = 1-acylglycerophosphocholine + a sterol ester

3.2 Amino Acid Sequence

The amino acid sequence of this GCAT is identical to the amino acid sequence of the native GCAT except for modification at one amino acid, asparagine at position 80 has been changed to aspartic acid. The amino acid sequence of the GCAT is shown in Appendix 1.

3.3 GCATs Safety

Aeromonas salmonicida is a particularly virulent pathogen of salmonid fish. A secreted GCAT has in the past been considered as key virulence determinant of this bacterium[9], like the GCAT of *Legionella pneumophila* was suggested to be involved in *Legionella* pathogenicity[10]. However, recent research using mutant strains has shown that GCAT is not required for intracellular infection of *L.* pneumophila and likely has no role in cytopathogenicity[11]. According to the WHO, *A. salmonicida* is a fish pathogen, but has not been associated with human infection[12].

There are several published works examining the toxicity of GCAT (KLM3') from *Aeromonas salmonicida* on erythrocytes from both fish and mammalian sources[9,13,14] Buckley et al.[14] demonstrated that while the individual glycerophospholipids found in human erythrocytes could serve as substrates for GCAT *in vitro*, there was no difference in the hemolysis rate between erythrocytes treated with GCAT 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. [13] states that proteolytic activation of pro-GCAT results in lysis of fish erythrocytes although not directly of mammalian erythrocytes and cites 2 additional references supporting this statement[15,16].

Lee and Ellis[9] provide a rationale for the specificity of GCAT for fish over mammalian erythrocytes. The preferred substrate for GCAT is phosphatidylcholine substituted with unsaturated fatty acids[14] 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 GCAT is observed mainly in *in vitro* assays and the intact mammalian erythrocytes are not lysed. Further, the hemolytic effect of GCAT is highly specific. Fish erythrocytes are highly susceptible to lysis by GCAT due to a much higher percentage of phosphatidylcholine (2x) than that found in mammalian erythrocytes. This evidence strongly suggests that GCAT is not a hemolysin for mammalian erythrocytes.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the KLM3' GCAT which follows standard industry practice.[20-22] For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with current FDA's Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the GCAT enzyme concentrate are standard ingredients used in the enzyme industry.[20-22] All the raw materials conform to the specifications of the Food Chemicals Codex, 5th edition, 2003 ("FCC"),[23] 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.

The antifoam used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoams in the production process is $\leq$1.5%.

4.2 Fermentation Process

The GCAT is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Section 2. 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.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 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.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units of bacteria or fungi other than the production strain are present at levels $>10^3$CFU/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both concentration and formulation processes.

4.3.1 Concentration process

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation
2. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration

4.3.2 Formulation and standardization process

The product is stabilized with glycerol and potassium sorbate and sold as a liquid.

4.3.3 Quality control of finished product

The final GCAT preparation from *B. licheniformis* is analyzed 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 ("JEFCA")[24] in 2006 and the FCC[23]. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The GCAT enzyme preparation has the following typical composition:

Activity: 1050-1150 U/g (spec: >1000 LATU/g)
Glycerol: 33-38 wt% Glycerol
Potassium sorbate: 0.5 wt%.

5.2 Specifications

The GCAT enzyme preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC. See FCC (2003)[23] at page 149. In addition, the GCAT enzyme preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA).[24]

The following specifications have been established for the GCAT enzyme:

(See next page)

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Property	Method Number	Reference Method	Specification
ENZYME ACTIVITIES			
Phospholipase	959W	Genencor Method	>1000LATU/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

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Property	Method Number	Reference Method	Specification
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			
Heavy Metals, as Pb	603W	FAO Food and Nutrition Paper No. 5, GUIDE TO SPECIFICA-TION, General notices, General analytical techniques, Identification tests; Test solutions, and other reference materials, 1983, 2nd revision	Less than 30 mg/kg
Arsenic	603W-AS	Same as Heavy Metals as Lead	Less than 3 mg/kg
Cadmium	603W-CD	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Mercury	603W-HG	Same as Heavy Metals as Lead	Less than 0.5 mg/kg
Lead	603W-PB	Same as Heavy Metals as Lead	Less than 5 mg/kg

Heavy metals, lead, arsenic, cadmium, mercury, antibacterial activity, and mycotoxins are analyzed at regular intervals. Activity and microbial specifications are analyzed on every production batch of enzyme.

The lead, Coliforms and Salmonella specifications meet FCC and JEFCA requirements. The *E. coli*, antibacterial activity and mycotoxin specifications meet JEFCA requirements and are not included in FCC (although FCC mentions mycotoxins, but has not established tolerances.) The production microorganism specification is a Danisco specification and is not mentioned in FCC or JEFCA.

The GCAT activity assay is based on a 10-minute reaction of the enzyme with phosphatidylcholine. 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.

6. APPLICATION

6.1 Mode of Action

The effectiveness of this GCAT 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).

The enzyme preparation will be used in egg yolk and whole eggs, in processed meats, in degumming of vegetable oils, in milk products like cheese, and in bakery products containing eggs like cake products.

6.2 Use Levels

6.2.1 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.

The enzyme preparation will be added to eggs for mayonnaise production at a maximum of 5000 LATU/kg food.

6.2.2 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.

The enzyme preparation will be added to processed meats at 300LATU/kg food.

6.2.3 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.

The enzyme preparation will be added to oil for degumming at a maximum of 1000 LATU/kg food.

6.2.4 Milk products

The enzyme preparation will be used in milk products. It will contribute to increased yield during cheese production. The enzyme added to milk will convert milk phospholipids to lysophospholipids. Because of the emulsification properties of lysophospholipids, this will contribute to increased cheese yield by entrapping more lipid in the cheese curd.

By the action of the GCAT in milk, lysolecithin is produced by transfer reaction of fatty acid from phospholipids to cholesterol during formation of cholesterol ester. Cholesterol ester is not an unknown constituent of the food diet as 8-15 % of the cholesterol in the diet is available as cholesterol esters.

The enzyme preparation will be added in cheese manufacture at a maximum of 1000 LATU/kg food.

6.2.5 Bakery products

In cakes enzymatically modified egg yolk gives a softer and more tender crumb.

The enzyme preparation will be added to eggs used in bakery products at a maximum of 500 LATU/kg food.

6.3 Enzyme Residues in the Final Foods

The enzyme is expected to be removed during the subsequent production processes for all but the cheese application. During oil processing, most of the enzyme will separate from the oil 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. 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 containing the whey proteins is not cooked. Residual enzyme in the cheese will also be active, unless the cheese is heated.

7. SAFETY EVALUATION

7.1 Safety of 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. [2] 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.[3] Pariza and Foster[2] define a non-toxigenc 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.

7.1.1 Safety of 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 four 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 licheniformis* 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), and
- Alpha-amylase derived from *Bacillus licheniformis* carrying a gene encoding a modified alpha-amylase from *Bacillus licheniformis* (GRN No. 79).

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[17].

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 proteases and pullulanase (Arrêté du 05/09/1989 et compléments).

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 by de Boer, et al[18].

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 Fifth 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, have been used by Genencor for the production of α-amylase enzyme preparations since 1989, as well as for the production of proteases, pullulanase and xylanase.

Numerous feeding, mutagenicity and carcinogenicity studies using enzyme products from *B. licheniformis* BRA7-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

7.1.2 Safety of the donor source

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 GCAT has in the past been considered as key virulence determinant of this bacterium[9], like the GCAT of *Legionella pneumophila* was suggested to be involved in *Legionella* pathogenicity [10]. However, recent research using mutant strains has shown that GCAT is not essential for intracellular infection of *L. pneumophila* [11]. According to the WHO, *A. salmonicida* is a fish pathogen, but has not been associated with human infection [12].

There are several published works examining the toxicity of GCAT (KLM3') from *Aeromonas salmonicida* on erythrocytes from both fish and mammalian sources[9,13,14]. Buckley et al. [14] demonstrated that while the individual glycerophospholipids found in human erythrocytes could serve as substrates for GCAT *in vitro*, there was no difference in the hemolysis rate between erythrocytes treated with GCAT 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., [13] states that proteolytic activation of pro-GCAT results in lysis of fish erythrocytes although not directly of mammalian erythrocytes and cites 2 additional references supporting this statement [15,16].

Lee and Ellis, [9] provide a rationale for the specificity of GCAT for fish over mammalian erythrocytes. The preferred substrate for GCAT is phosphatidylcholine substituted with unsaturated fatty acids[14] 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 GCAT is observed mainly in *in vitro* assays and the intact mammalian erythrocytes are not lysed. Further, the hemolytic effect of GCAT is highly specific. Fish erythrocytes on the other hand, are highly susceptible to lysis by GCAT due to a much higher percentage of phosphatidylcholine (2x) than that found in mammalian erythrocytes. This evidence strongly suggests that GCAT is not a hemolysin for mammalian erythrocytes.

7.2 Safety of GCAT

7.2.1 Allergenicity

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 functions. 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.

Because exposure to enzymes is very low, even if they were potentially allergenic by the oral route, the likelihood of allergic sensitization of consumers to these proteins is virtually nil.

7.3 Safety of the Manufacturing Process

The manufacturing process for the production of GCAT will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, filtration and formulation, resulting in a liquid GCAT enzyme preparation. In addition, a dry product is also produced; it is dried and agglomerated using any one of the common drying methods, such as spray drying, fluid bed agglomeration or fluid bed spray drier. The process, described in Section 4, is conducted in accordance with FDA's cGMPs as set forth in 21 C.F.R. Part 110. The resultant products meet the general requirements for enzyme preparations of the FCC[23] and WHO/JEFCA[24].

7.4 Safety Studies

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

Study	Test Item
Acute dermal irritation study in rabbits (sequential approach)	UF concentrate
Acute Eye Irritation/Corrosion Study in the Rabbit	UF concentrate
Acute oral toxicity in rats – Fixed dose procedure	UF concentrate
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

7.4.1 Test item preparations

Test items for the studies, as indicated above, were prepared as follows:

7.4.1.1 UF Concentrate

UF concentrate is the ultrafiltered concentrate of the enzyme preparation, stopped during the manufacturing process before the addition of formulation ingredients. The UF concentrate is characterized by:

Enzyme activity	1156 LATU/ml
Moisture	90.28%
Ashes	1.05%
%TOS (100 –%ash-%moisture)	8.67%
Total Protein content	30.40%

The UF concentrate was stored frozen in aliquots and thawed before use.

7.4.1.2 Lyophilized UF Concentrate

The test item was made by lyophilizing the UF concentrate. It is characterized by:

Enzyme activity	21512 LATU/ml
Dry matter content	89%

7.4.1.3 Acute dermal irritation study in rabbits (sequential approach)

A. Procedure:
The objective of this study is to assess the local dermal irritant effect of the enzyme. This study was conducted according to the method recommended in the OECD Guideline No. 404 and EEC Directive Annex I, II, III and IV, Official Journal of the European Communities published in 1993.

In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and reading was made at 1, 24, 48 and 72 hours post application.

B. Results

No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays.

C. Evaluation

The mean score for skin edema and erythema was 0.0. According to the Directive of the Commission 93/21/EEC of April 27, 1993, this GCAT enzyme is not a skin irritant.

7.4.1.4 Acute Eye Irritation/Corrosion Study in the Rabbit

A. Procedure

The objective of this study is to assess the ocular irritation potential of the enzyme. This study was conducted according to the method recommended in the OECD Guideline No. 405 and EEC Directive, part B5, Official Journal of the European Communities published in 1992.

In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded 1 and 24 hours later. The right eye served as control. After the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage at 48 and 72 hours after the treatment. A confirmatory test was conducted with 2 rabbits.

B. Results

In the initial study, slight discharge was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay with 2 rabbits, one animal had discharge at the 1-hour examination and conjunctiva redness (+1) which persisted at the 24-hour examination period. At all other examinations, all animals appeared normal.

C. Evaluation

The primary eye irritation score was 0.1. According to the EEC Directive published in the Official Journal of the European Communities, No. L 383A, volume 35, 29.12.1992, part B5, this GCAT enzyme should not be classified as an eye irritant.

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7.4.1.5 Acute oral toxicity in rats – Fixed dose procedure

A. Procedure:

The objective of this study is to assess the acute toxicity of the enzyme 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).

B. 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.

C. 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).

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

A. Procedure

The objective of this study is to investigate the potential of the enzyme to induce systemic toxicity after repeated daily oral administration to SPF Sprague Dawley rats (b(4)) 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 histopathological 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.

B. 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 the enzyme 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 deaths
Group 2 (low dose): No deaths
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 the enzyme 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 histopathological examinations.

C. 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).

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7.4.1.7 Bacterial Reverse Mutation Assay – Ames assay

A. Procedure:

The objective of this assay is to assess the potential of the enzyme 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.

B. Results:

A dose-related amount of insoluble material was observed on all plates. The enzyme 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. The enzyme 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 this GCAT enzyme 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.

C. Evaluation

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

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

7.4.1.8 *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

A. Procedure

The objective of this assay is to investigate the potential of the enzyme to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). The enzyme 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 the enzyme 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:

a. 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.

b. Metaphase analysis (i.e. evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.

c. 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.

B. 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).

The enzyme 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.

C. Evaluation

Under the conditions of this test, the lyophilized powder did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation. However, the enzyme was extremely cytotoxic to the lymphocytes in cultures. The cytotoxic effect is related to the mechanism of action of an acyltransferase enzyme. Indeed, the enzyme 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 the enzyme 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.

7.4.1.8 *In* vivo Mouse Micronucleus Test

A. Procedure

The objective of this study is to determine the potential of the enzyme 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 b(4)) 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 the enzyme 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.

B. 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 concluded to be a dosing/gavaging error, due to the presence of blood in the oral and nasal cavities.

The main test was performed using male mice only, because 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 enzyme groups. The positive control produced a significant increase in micronuclei PCE, substantiating the sensitivity and validity of the assay.

C. 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).

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7.5 Overall Safety Assessment and Conclusions

The GCAT enzyme is not an irritant to the eyes and skin. According to the Directive of the Commission 93/21/EEC of April 27, 1993, the enzyme is non hazardous based on acute oral and irritation studies. In genotoxicity studies, the enzyme is not mutagenic, clastogenic or aneugenic. Daily oral administration of the enzyme 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.

7.5.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, 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

7.5.2 Human Exposure Assessment

The GCAT enzyme provides benefits in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat.

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	
Cheese	0.27	1000	0.27	
Processed meat	0.67	300	0.20	
TOTAL for US			**1.45**	**0.039**
Danish Population				
Oils	0.83	1000	0.83	
Eggs/bakery	0.17	500	0.09	
Eggs/mayonnaise	0.02	5000	0.10	

Cheese	0.52	1000	0.52	
Processed meat	0.70	300	0.21	
TOTAL for DK			**1.75**	**0.047**

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

Estimated Daily Intake of Acyltransferase BL1

The estimated intake of this GCAT 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[19]. The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007).

The maximum estimated daily intake of the enzyme for the US and Danish population is 0.039 and 0.047 mg total protein/kg bw/day, respectively, and these estimates represent a worst case scenario.

The maximum estimated daily intake of the enzyme from degumming of oil in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products is 0.047 mg total protein/kg bw/day under the scenario that (1) all above commodities are treated with the enzyme, (2) 100% of the enzyme remains in the product after processing, and (3) all consumers eat all these commodities treated with the enzyme. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing (see discussion in 6.2).

Maximum Estimated Daily Intake of the GCAT enzyme = 0.047 mg/kg bw

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, in cheese and in processed meat products (i.e., cumulative risk), the NOAEL of 41 mg total protein/kg bw/day still offers an 872X fold margin of safety and the enzyme preparation is safe for these intended uses.

7.6 Conclusion

The safety of this GCAT enzyme 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 is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. The enzyme is not an eye and skin irritant and is not acutely toxic. A battery of genotoxicity assays was conducted and under the conditions of these assays the enzyme is not

a mutagen, not a clastogen, not an aneugen and does not increase the formation of micronuclei in bone marrow erythrocytes. Although the enzyme demonstrates cytotoxicity in *in vitro* cultures, no similar adverse effects are found in the whole animal. Daily administration of the enzyme 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).

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, enzyme preparations derived from *Bacillus licheniformis*, including α-amylase, protease, pullulanase, and xylanase, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as Genencor's own unpublished safety studies, support such a conclusion.

Bacillus licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. An enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nonpathogenic and non-toxigenic; 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. Information is provided in this document to support that the components of the enzyme preparation meet these standards.

The safety studies conducted by Genencor established a NOAEL at 41.0 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day.

Utilizing the information provided, the enzyme preparation was taken through the Pariza-Johnson decision tree for microbial enzymes (see Appendix 3) and the preparation was determined to be acceptable for use in foods as described above.

Based on the available data from the literature and generated by Genencor, the company has concluded that the enzyme preparation from *Bacillus licheniformis* strain 3265 is safe and suitable for use in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products.

In addition, the safety determination including construction of the production organism, the production process and materials, and safety of the product were reviewed by Dr. Michael W. Pariza, Dr. Herbert Blumenthal and Dr. Joseph F. Borzelleca and they concurred with the company's conclusion that the product is GRAS (see Appendix 4). The panel was provided with:

- 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[1].

The panel independently critically evaluated these documents and other materials deemed relevant. The panel concluded that the GCAT meeting the described specifications and produced in compliance with cGMP is GRAS by scientific procedures for used in foods as described by Genencor.

GENENCOR

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10. LIST OF APPENDICES

Appendix 1 – The amino acid sequence of GCAT

Appendix 2 - The Manufacturing process

Appendix 3 – The Pariza/Johnson Decision Tree

Appendix 4 – GRAS Panel Report

1

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Appendix 1 – The amino acid sequence of GCAT

Protein sequence comparison of this GCAT to wild type *Aeromonas salmonicida* subsp. *salmonicida* GCAT (gi|39028). Only the mature parts are aligned. Residues identical in the two amino acid sequences are represented by a dot, and changes are indicated in red font.

```
Reference molecule:   gi|39028|emb|CAA50021.1|    19 -   335   (  317 aa)  Homology

       Sequence  2:   KLM3'\(mat          1 -   317   (  317 aa)     100%

Alignment type:       Local (FastScan)
Homology details:     Percent Max 99; Score 631; Length 317; Conserv N


gi|39028|emb (    19) adtrpafsrivmfgdslsdtgkmyskmrgylpsspyyegrfsngpvwleqltkqfpglt
KLM3'\(mat   (     1) ............................................................

gi|39028|emb (    79) ianeaeggatavaynkiswnpkyqvinnldyevtqflqkdsfkpddlvilwvgandylay
KLM3'\(mat   (    61) ...................d......................................

gi|39028|emb (   139) gwnteqdakrvrdaisdaanrmvlngakqillfnlpdlgqnpsarsqkvveavshvsayh
KLM3'\(mat   (   121) ............................................................

gi|39028|emb (   199) nkllinlarqlaptgmvklfeidkqfaemlrdpqnfglsdvenpcydggyvwkpfatrsv
KLM3'\(mat   (   181) ............................................................

gi|39028|emb (   259) stdrqlsafspgerlaiagnpllaqavaspmarrsasplnceqkmfwdqvhpttvvhaal
KLM3'\(mat   (   241) ............................................................

gi|39028|emb (   319) seraatfietqyeflah
KLM3'\(mat   (   301) ................
```

2

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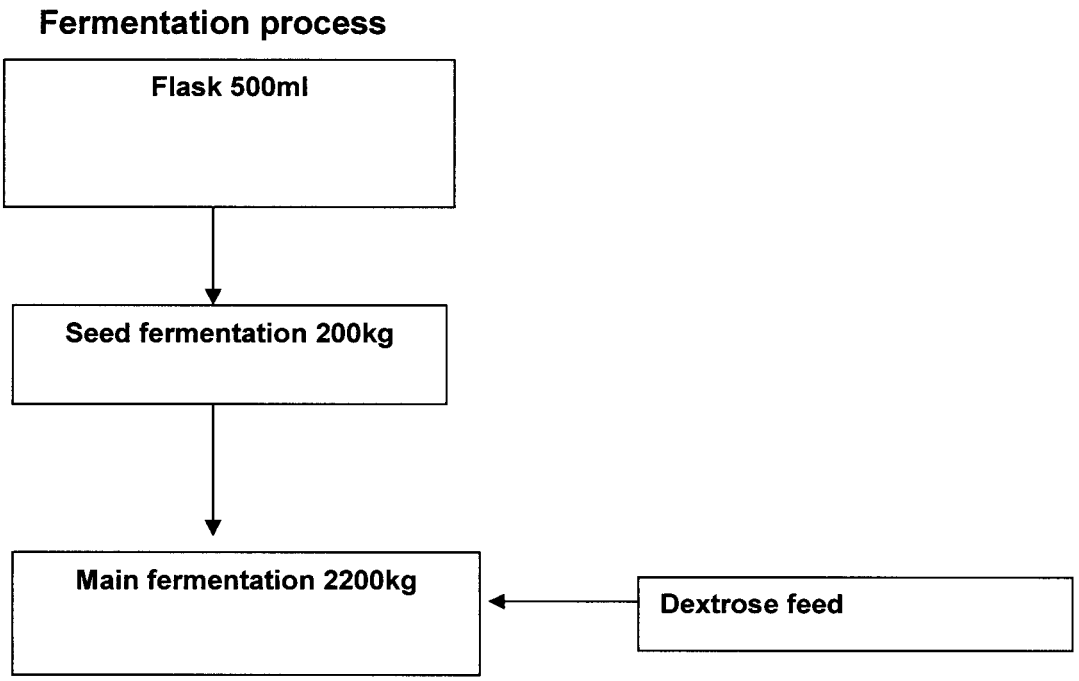
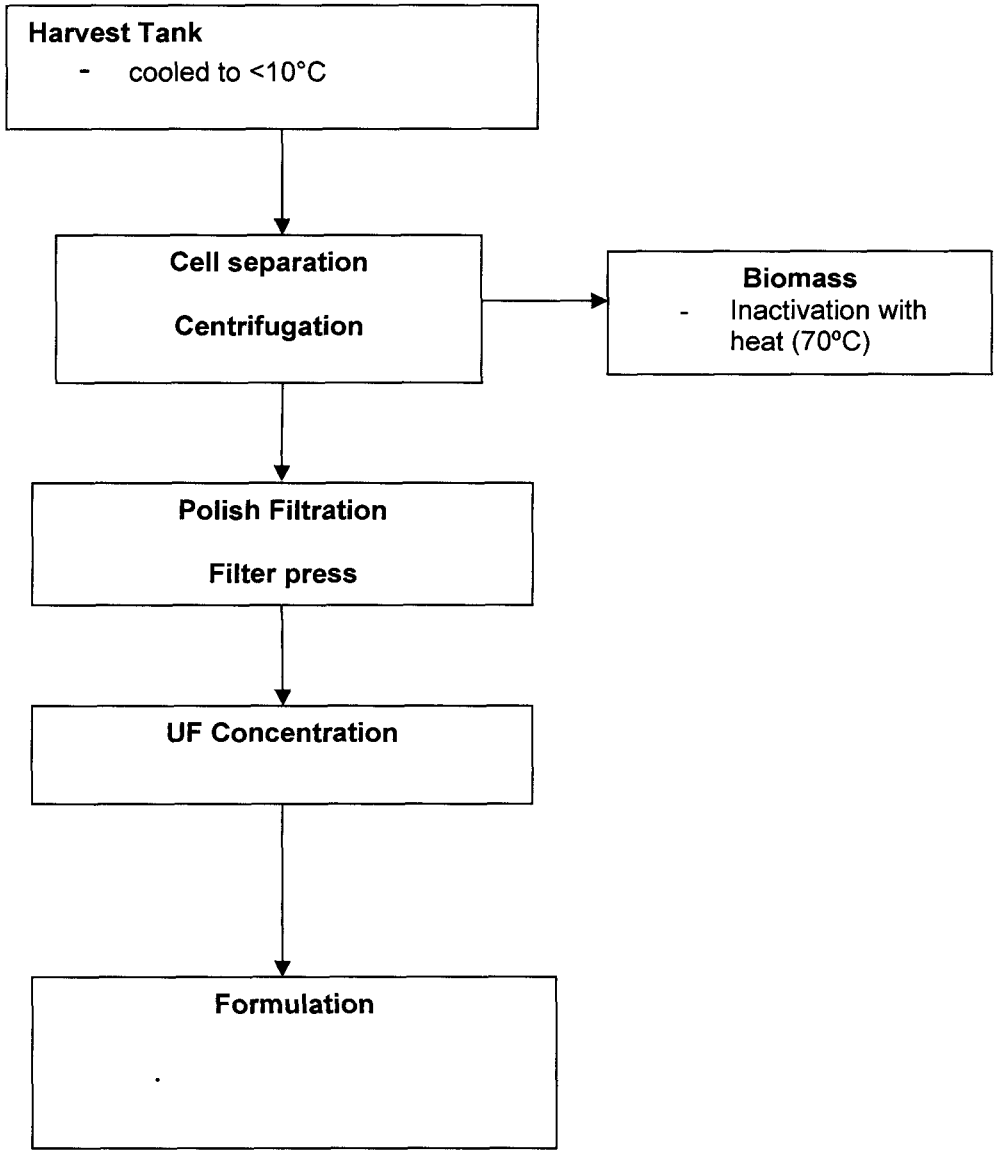
Appendix 2 - The Manufacturing process



GENENCOR

Recovery flow diagram



3

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Appendix 3 – 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

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Appendix 4 – GRAS Panel Report

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') 29 January 2008

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'.

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. A panel was assembled by Danisco USA 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.

Commercial enzyme preparations that are used in food processing 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 manufacturing process, e.g., components of the fermentation media or the residues of processing aids.

Danisco USA provided the Expert Panel with a dossier containing 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. They then spoke with representatives of Danisco USA and the Genencor Division via teleconference on December 4, 2007, 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; and
- To convert milk phospholipids to lysophospholipids, improving emulsification and increasing cheese yield by entrapping more lipid in the cheese curd.

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 *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 of 0.039 mg total protein/kg bw/day in the US and 0.047 mg total protein /kg bw/day in Denmark.

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).

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 stated that the host organism has a long history of safe industrial use and conclude 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 that the components of the enzyme preparation meet these standards.

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 studies are available from Danisco's Regulatory Affairs department in Palo Alto, CA. Danisco concludes that the results of the toxicity and mutagenicity tests demonstrate the safety of the Danisco's glycerophospholipid cholester acyltransferase KLM3' preparation and support the safe use of enzyme preparations produced by the production strain.
A NOAEL is established at 41.0 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day), the highest dose tested.

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 in a dossier 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, areGenerally Recognized As Safe (GRAS) based on scientific procedures.

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

Feb. 5, 2008

Herbert Blumenthal, PhD
Consultant

05 February 2008

Joseph F. Borzelleca, PhD
Professor of Pharmacology and Toxicology
Medical College of Virginia

09 February 2008

Michael W. Pariza, PhD
Director, Food Research Institute
Wisconsin Distinguished Professor of Food Microbiology and Toxicology
University of Wisconsin - Madison

SUBMISSION END

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FEB 4 2009
BY... (b)(6)

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A Danisco Division

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FEB 2 2009

February 2, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRSN 000265

Dear Dr. Martin:

Please accept the enclosed amendment to the above referenced GRAS Notice.

If you have questions or require additional information, please contact me at 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b)(6)

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures (3 binders)

[redacted]

GENENCOR

Glycerophospholipid Cholesterol Acyltransferase (GCAT) Enzyme Preparation from *Bacillus licheniformis* Expressing the GCAT Gene from *Aeromonas salmonicida subsp. salmonicida* Is Generally Recognized As Safe For Use in Food Processing

Notification Submitted by Genencor, a Danisco Division

November 3, 2008
(Amended February 2, 2009)

GENENCOR

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1. GENERAL INTRODUCTION

FoodPro™ LysoMax® Oil is the Danisco trade name used for the GCAT enzyme preparation produced by submerged fermentation of *Bacillus licheniformis* carrying the gene encoding GCAT from *Aeromonas salmonicida subsp. salmonicida*, also called KLM3'. DNA encoding the GCAT protein was synthesized according to the known DNA sequence, modified at one amino acid position, and codon optimized for expression in *B. licheniformis,* and inserted into *B. licheniformis.*

The enzyme is intended 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; to improve the emulsification of processed meat products and contribute to better consistency and reduced cooking loss; for the degumming of vegetable oils; to increase yield in milk products like cheese; and to give a softer and more tender crumb in bakery products containing eggs.

The enzyme is a glycerophospholipid cholesterol acyltransferase (GCAT) (IUBMB 2.3.1.43) which catalyzes phosphatidylcholine + a sterol to 1-acylglycerophosphocholine + a sterol ester.

Pursuant to the regulatory and scientific procedures established in proposed 21 C.F.R. 170.36, Danisco has determined that its GCAT enzyme preparation from a modified strain of *Bacillus licheniformis* expressing the GCAT gene from *Aeromonas salmonicida subsp. salmonicida* is a Generally Recognized As Safe ("GRAS") substance for the intended food application and is, therefore, exempt from the requirement for premarket approval. The information provided in the following sections is the basis of our determination of GRAS status of a GCAT enzyme preparation produced by a *B. licheniformis* host, which has been modified to express a gene encoding GCAT from *Aeromonas salmonicida subsp. salmonicida.*. Our safety evaluation in Section 7 includes an evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for food use.[1,2] The safety of the production organism for this GCAT, *B. licheniformis,* is discussed in Sections 2 and 7. Another essential aspect of the safety evaluation of enzymes derived from genetically modified microorganisms is the identification and characterization of the inserted genetic material.[3-8] The genetic modifications used to construct this production organism are well defined and are described in Section 2. The safety evaluation described in Section 7 shows no evidence to indicate that any of the cloned DNA sequences and incorporated DNA code for or express a harmful toxic substance.

GENENCOR

1.1 Name and Address of Notifier

Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304

1.2 Common or Usual Name of Substance

GCAT enzyme preparation from *B. licheniformis* expressing the gene encoding GCAT enzyme from *Aeromonas salmonicida* subsp. *salmonicida.*

1.3 Applicable Conditions of Use

The GCAT enzyme is intended 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; to improve the emulsification of processed meat products and contribute to better consistency and reduced cooking loss; for the degumming of vegetable oils; to increase yield in milk products like cheese; and to give a softer and more tender crumb in bakery products containing eggs. The enzyme preparation is used at minimum levels to achieve the desired effect and according to requirements of normal production following current Good Manufacturing Practices. It is expected that mayonnaise, processed meats, cheese, degummed oils and bakery products produced using this GCAT will be consumed by the general population.

1.4 Basis for GRAS Determination

This GRAS determination is based upon scientific procedures.

1.5 Availability of Information for FDA Review

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this notice. The package includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.

2. PRODUCTION MICROORGANISM

2.1 Production Strain

The production organism of the GCAT enzyme preparation is *B. licheniformis* strain GICC03265. It is derived by recombinant DNA methods from a strain of *Bacillus licheniformis* modified to express a synthetic GCAT gene DNA that has been modified at one amino acid and codon optimized for expression in *B. licheniformis* (see Section 2.2). *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[1] and several expert groups.[2-8] It contains the synthetic GCAT gene under the regulation of a native *B. licheniformis* promoter and terminator along with a selectable marker, the native *B. licheniformis cat* gene.

The inserted DNA was integrated into the host on a in a vector derived from *Bacillus* plasmids pUB110 and pE194. The introduced DNA integrated into the Bacillus chromosome at the *cat* locus by Campbell type recombination. After integration all vector sequences of the plasmid were deleted by recombination between direct repeated *cat* sequences.

2.2 Host Microorganism

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 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 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 GCAT Expression 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 GCAT gene) is located between these repeats.

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.

2.4 Stability of the Introduced Genetic Sequences

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

2.5 Antibiotic Resistance Gene

The construction of the production organism utilized the native chloramphenicol resistance (CAT) gene from *B. licheniformis*; no new antibiotic resistance was introduced.

2.6 Absence of Production Microorganism in Product

The absence of the production microorganism is an established specification for the commercial product at a detection limit of 1CFU/g. The production organism does not end up in food and therefore, the first step in the safety assessment as described by the IFBC[3] is satisfactorily addressed.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1 Enzyme Identity

Classification	Acyltransferase
IUBMB Nomenclature	phosphatidylcholine sterol O-acyltransferase
IUBMB Number:	2.3.1.43
CAS Number:	9031-14-5
EINECS Number:	232-796-2
Reaction catalyzed	phosphatidylcholine + a sterol = 1-acylglycerophosphocholine + a sterol ester

3.2 Amino Acid Sequence

The amino acid sequence of this GCAT is identical to the amino acid sequence of the native GCAT except for modification at one amino acid, asparagine at position 80 has been changed to aspartic acid. The amino acid sequence of the GCAT is shown in Appendix 1.

3.3 GCATs Safety

Aeromonas salmonicida is a particularly virulent pathogen of salmonid fish. A secreted GCAT has in the past been considered as key virulence determinant of this bacterium[9], like the GCAT of *Legionella pneumophila* was suggested to be involved in *Legionella* pathogenicity[10]. However, recent research using mutant strains has shown that GCAT is not required for intracellular infection of *L.* pneumophila and likely has no role in cytopathogenicity[11]. According to the WHO, *A. salmonicida* is a fish pathogen, but has not been associated with human infection[12].

There are several published works examining the toxicity of GCAT (KLM3') from *Aeromonas salmonicida* on erythrocytes from both fish and mammalian sources[9,13,14] Buckley et al.[14] demonstrated that while the individual glycerophospholipids found in human erythrocytes could serve as substrates for GCAT *in vitro*, there was no difference in the hemolysis rate between erythrocytes treated with GCAT 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. [13] states that proteolytic activation of pro-GCAT results in lysis of fish erythrocytes although not directly of mammalian erythrocytes and cites 2 additional references supporting this statement[15,16].

Lee and Ellis[9] provide a rationale for the specificity of GCAT for fish over mammalian erythrocytes. The preferred substrate for GCAT is phosphatidylcholine substituted with unsaturated fatty acids[14] 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 GCAT is observed mainly in *in vitro* assays and the intact mammalian erythrocytes are not lysed. Further, the hemolytic effect of GCAT is highly specific. Fish erythrocytes are highly susceptible to lysis by GCAT due to a much higher percentage of phosphatidylcholine (2x) than that found in mammalian erythrocytes. This evidence strongly suggests that GCAT is not a hemolysin for mammalian erythrocytes.

4. MANUFACTURING PROCESS

This section describes the manufacturing process for the KLM3' GCAT which follows standard industry practice.[20-22] For a diagram of the manufacturing process, see Appendix 2. The quality management system used in the manufacturing process complies with the requirements of ISO 9001. The enzyme preparation is also manufactured in accordance with current FDA's Good Manufacturing Practices ("cGMP") as set forth in 21 C.F.R. Part 110.

4.1 Raw Materials

The raw materials used in the fermentation and recovery process for the GCAT enzyme concentrate are standard ingredients used in the enzyme industry.[20-22] All the raw materials conform to the specifications of the Food Chemicals Codex, 6th edition, 2008 ("FCC"),[23] 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.

The antifoam used in the fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 24, 1998. The maximum use level of these antifoams in the production process is $\leq$1.5%.

4.2 Fermentation Process

The GCAT is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. licheniformis* described in Section 2. 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.

4.2.1 Production organism

A new lyophilized stock culture vial of the *B. licheniformis* production organism described in Section 2 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.

4.2.2 Criteria for the rejection of fermentation batches

Growth characteristics during fermentation are observed microscopically. Samples are taken from each fermentation stage (inoculum, seed and main fermenter) before inoculation, at regular intervals during growth and before harvest or transfer. These samples are tested for microbiological contamination by plating on a nutrient medium.

A fermentation batch is declared as 'contaminated' if colony forming units of bacteria or fungi other than the production strain are present at levels $>10^3$CFU/ml.

If a fermentation batch is determined to be contaminated, it will be rejected if deemed appropriate. If the contamination is minor and determined to be from common non-pathogenic environmental microbes, the fermentation may be processed.

4.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both concentration and formulation processes.

4.3.1 Concentration process

The enzyme is recovered from the culture broth by the following series of operations:

1. Primary separation –centrifugation
2. Polish filtration – for removal of residual production strain organisms and as a general precaution against microbial degradation
3. Concentration – ultrafiltration

4.3.2 Formulation and standardization process

The product is stabilized with glycerol and potassium sorbate and sold as a liquid.

4.3.3 Quality control of finished product

The final GCAT preparation from *B. licheniformis* is analyzed 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 ("JEFCA")[24] in 2006 and the FCC[23]. These specifications are set forth in Section 5.

5. COMPOSITION AND SPECIFICATIONS

5.1 Quantitative Composition

The GCAT enzyme preparation has the following typical composition:

Activity: 900-1100 LATU/g equal to maximum of 82.9 mg TOS/g
Glycerol: 33-38 % w/w
Potassium sorbate: 0.5 w/w.

GENENCOR

5.2 Specifications

The GCAT enzyme preparation conforms to the general and additional requirements for enzyme preparations as described in the FCC. See FCC (2008)[23] under Enzyme Preparations. In addition, the GCAT enzyme preparation also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Join FAO/WHO Expert Committee on Food Additives (JEFCA).[24]

The following specifications have been established for the GCAT enzyme:

(See next page)

GENENCOR

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

Lead and antibacterial activity are analyzed at regular intervals. Activity and microbial specifications are analyzed on every production batch of enzyme.

The lead, Coliforms and Salmonella specifications meet FCC and JEFCA requirements. The *E. coli* and antibacterial activity specifications meet JEFCA requirements and are not included in FCC. The production microorganism specification is a Danisco specification and is not mentioned in FCC or JEFCA.

The GCAT activity assay is based on a 10-minute reaction of the enzyme with phosphatidylcholine. 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 activity of GCAT is defined in LATU. 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.

6. APPLICATION

6.1 Mode of Action

The effectiveness of this GCAT 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).

The enzyme preparation will be used in egg yolk and whole eggs, in processed meats, in degumming of vegetable oils, in milk products like cheese, and in bakery products containing eggs like cake products.

6.2 Use Levels

6.2.1 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.

The enzyme preparation will be added to eggs for mayonnaise production at a maximum of 5000 LATU/kg food equal to 376.9 mg TOS/kg food.

6.2.2 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.

The enzyme preparation will be added to processed meats at 300LATU/kg food equal to 22.6 mg TOS/kg food.

6.2.3 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.

The enzyme preparation will be added to oil for degumming at a maximum of 1000 LATU/kg food equal to 75.4 mg TOS/kg food.

6.2.4 Milk products

The enzyme preparation will be used in milk products. It will contribute to increased yield during cheese production. The enzyme added to milk will convert milk phospholipids to lysophospholipids. Because of the emulsification properties of lysophospholipids, this will contribute to increased cheese yield by entrapping more lipid in the cheese curd.

By the action of the GCAT in milk, lysolecithin is produced by transfer reaction of fatty acid from phospholipids to cholesterol during formation of cholesterol ester. Cholesterol ester is not an unknown constituent of the food diet as 8-15 % of the cholesterol in the diet is available as cholesterol esters.

The enzyme preparation will be added in cheese manufacture at a maximum of 1000 LATU/kg food equal to 75.4 mg TOS/kg food.

6.2.5 Bakery products

In cakes enzymatically modified egg yolk gives a softer and more tender crumb.

The enzyme preparation will be added to eggs used in bakery products at a maximum of 500 LATU/kg food equal to 37.7 mg TOS/kg food.

6.3 Enzyme Residues in the Final Foods

The enzyme is expected to be removed during the subsequent production processes for all but the cheese application. During oil processing, most of the enzyme will separate from the oil 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. 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 containing the whey proteins is not cooked. Residual enzyme in the cheese will also be active, unless the cheese is heated.

7. SAFETY EVALUATION

7.1 Safety of 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. [2] 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.[3] Pariza and Foster[2] define a non-toxigenc 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.

7.1.1 Safety of the host

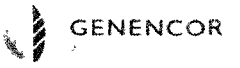
Bacillus licheniformis is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium, and is also considered as GILSP worldwide.

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 four 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 licheniformis* 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), and
- Alpha-amylase derived from *Bacillus licheniformis* carrying a gene encoding a modified alpha-amylase from *Bacillus licheniformis* (GRN No. 79).

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[17].

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 proteases and pullulanase (Arrêté du 05/09/1989 et compléments).



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 by de Boer, et al[18].

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 Fifth 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, have been used by Genencor for the production of α-amylase enzyme preparations since 1989, as well as for the production of proteases, pullulanase and xylanase.

Numerous feeding, mutagenicity and carcinogenicity studies using enzyme products from *B. licheniformis* BRA7-derived strains have been performed, and no evidence of a toxic or mutagenic effect has been observed.

7.1.2 Safety of the donor source

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 GCAT has in the past been considered as key virulence determinant of this bacterium[9], like the GCAT of *Legionella pneumophila* was suggested to be involved in *Legionella* pathogenicity [10]. However, recent research using mutant strains has shown that GCAT is not essential for intracellular infection of *L. pneumophila* [11]. According to the WHO, *A. salmonicida* is a fish pathogen, but has not been associated with human infection [12].

There are several published works examining the toxicity of GCAT (KLM3') from *Aeromonas salmonicida* on erythrocytes from both fish and mammalian sources[9,13,14]. Buckley et al. [14] demonstrated that while the individual glycerophospholipids found in human erythrocytes could serve as substrates for GCAT *in vitro*, there was no difference in the hemolysis rate between erythrocytes treated with GCAT 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., [13] states that proteolytic activation of pro-GCAT results in lysis of fish erythrocytes although not directly of mammalian erythrocytes and cites 2 additional references supporting this statement [15,16].

Lee and Ellis, [9] provide a rationale for the specificity of GCAT for fish over mammalian erythrocytes. The preferred substrate for GCAT is phosphatidylcholine substituted with unsaturated fatty acids[14] 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 GCAT is observed mainly in *in vitro* assays and the intact mammalian erythrocytes are not lysed. Further, the hemolytic effect of GCAT is highly specific. Fish erythrocytes on the other hand, are highly susceptible to lysis by GCAT due to a much higher percentage of phosphatidylcholine (2x) than that found in mammalian erythrocytes. This evidence strongly suggests that GCAT is not a hemolysin for mammalian erythrocytes.

7.2 Safety of the Manufacturing Process

The manufacturing process for the production of GCAT will be conducted in a manner similar to other food and feed production processes. It consists of a pure-culture fermentation process, cell separation, concentration, filtration and formulation, resulting in a liquid GCAT enzyme preparation. The process, described in Section 4, is conducted in accordance with FDA's cGMPs as set forth in 21 C.F.R. Part 110. The resultant products meet the general requirements for enzyme preparations of the FCC[23] and WHO/JEFCA[24].

7.3 Safety of GCAT

7.3.1 Allergenicity

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[2] 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 [25]. Poulsen [26] 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.[27] 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 GCAT could be a food allergen was examined (for details, see Appendix 3). The full GCAT sequence was compared with known allergens using either the SDAP database (containing 737 allergens or the Allermatch database combined database containing 792 allergens. GCAT 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.[28]; Marknell DeWitt et al.[29]; Valenta et al.[30]), sunflower pollen (Asturias et al.[31]), and mugwort pollen (Wopfner et al.[32]), but not in any food allergens.

Further protein hydrophobicity analysis indicated this 6-amino acid stretch in the GCAT 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 GCAT does not match any known food allergens.

7.3.2 Safety Studies

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

Study	Test Item
Acute dermal irritation study in rabbits (sequential approach)	UF concentrate
Acute Eye Irritation/Corrosion Study in the Rabbit	UF concentrate
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

7.3.2.1 Test item preparations

Test items for the studies, as indicated above, were prepared as follows:

UF Concentrate

UF concentrate is the ultrafiltered concentrate of the enzyme preparation, stopped during the manufacturing process before the addition of formulation ingredients. The UF concentrate is characterized by:

Enzyme activity	1156 LATU/ml
Moisture	90.28%
Ashes	1.05%
%TOS (100 –%ash-%moisture)	8.67%
Total Protein content	30.40 mg/ml

The UF concentrate was stored frozen in aliquots and thawed before use.

Lyophilized UF Concentrate

The test item was made by lyophilizing the UF concentrate. It is characterized by:

Enzyme activity	21512 LATU/ml
Dry matter content	89%

7.3.2.2 Acute dermal irritation study in rabbits (sequential approach)

A. Procedure:
The objective of this study is to assess the local dermal irritant effect of the enzyme. This study was conducted according to the method recommended in the OECD Guideline No. 404 and EEC Directive Annex I, II, III and IV, Official Journal of the European Communities published in 1993.

In the initial test, the back of one rabbit was divided into 4 test sites. Three sites were used for test material application whereas the fourth test site served as control (vehicle only). All test sites were observed at 3 minutes and at 1 and 3 hours post application. A confirmatory test was conducted later with two rabbits and reading was made at 1, 24, 48 and 72 hours post application.

B. Results
No deaths or overt signs of toxicity were observed in this study. No effects on feed consumption and weight gain were recorded. No reactions were noted at any test site in both preliminary and confirmatory assays.

C. Evaluation
The mean score for skin edema and erythema was 0.0. According to the Directive of the Commission 93/21/EEC of April 27, 1993, this GCAT enzyme is not a skin irritant.

7.3.2.3 Acute Eye Irritation/Corrosion Study in the Rabbit

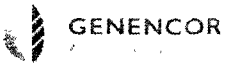
A. Procedure

The objective of this study is to assess the ocular irritation potential of the enzyme. This study was conducted according to the method recommended in the OECD Guideline No. 405 and EEC Directive, part B5, Official Journal of the European Communities published in 1992.

In the initial test, the test material was applied at 0.1 ml to the left eye and the grade of ocular reaction was recorded 1 and 24 hours later. The right eye served as control. After the 24-hour reading, fluorescein was instilled and then rinsed with 0.9% NaCl. The eye was then examined with an UV-light to detect corneal damage at 48 and 72 hours after the treatment. A confirmatory test was conducted with 2 rabbits.

B. Results

In the initial study, slight discharge was observed at the 1-hour observation period with clearing by 24 hours. In the confirmatory assay with 2 rabbits, one animal had discharge at the 1-hour examination and conjunctiva redness (+1)



which persisted at the 24-hour examination period. At all other examinations, all animals appeared normal.

C. Evaluation

The primary eye irritation score was 0.1. According to the EEC Directive published in the Official Journal of the European Communities, No. L 383A, volume 35, 29.12.1992, part B5, this GCAT enzyme should not be classified as an eye irritant.

7.3.2.4 Acute oral toxicity in rats – Fixed dose procedure

A. Procedure:

The objective of this study is to assess the acute toxicity of the enzyme 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).

B. 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.

C. 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).

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

A. Procedure

The objective of this study is to investigate the potential of the enzyme 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 histopathological 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.

B. 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 the enzyme 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 deaths
Group 2 (low dose): No deaths
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 the enzyme 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 histopathological examinations.

C. 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 ± 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).

7.3.2.6 Bacterial Reverse Mutation Assay – Ames assay

A. Procedure:

The objective of this assay is to assess the potential of the enzyme 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.

B. Results:

A dose-related amount of insoluble material was observed on all plates. The enzyme 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. The enzyme 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 this GCAT enzyme 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.

C. Evaluation

No biologically or statistically significant <u>increases</u> in the number of revertant colonies were noted after exposure to the enzyme.

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

7.3.2.7 *In vitro* Mammalian Chromosomal Aberration Test Performed with Human Lymphocytes

A. Procedure

The objective of this assay is to investigate the potential of the enzyme to induce numerical and/or structural changes in the chromosome of mammalian systems (i.e., human peripheral lymphocytes). The enzyme 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 the enzyme 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:

a. 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.
b. Metaphase analysis (i.e. evaluation of chromosomal aberration) was conducted on 100 metaphases per dose level.
c. 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.

B. 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).

The enzyme 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.

C. Evaluation

Under the conditions of this test, the lyophilized powder did not induce chromosomal aberrations (both structural and numerical) in mammalian cells both in the presence and absence of metabolic activation at the dose levels selected. However, toxicity (expressed as 50% reduction in mitotic index relative to the solvent control) was noted at higher dose levels and is related to the mechanism of action of GCAT, an acyltransferase enzyme. Indeed, the enzyme effectiveness is based on its ability to transfer acyl groups from phospholipids (phosphatidylcholine and phosphatidylethanolamine) and glycolipids to acceptor molecules such as cholesterol to form cholesterol acyl ester. In the absence of the acceptor molecule, cholesterol, a slow hydrolysis of the phospholipids will occur, giving rise to free fatty acids, lyso-phosphatidylcholine and lyso-phosphatidylethanolamine. These lyso derivatives are surface active reagents or detergents. In an *in vitro* assay such as the chromosomal aberration assay with human lymphocytes, GCAT slowly hydrolyzes lymphocyte membrane phospholipids due to the low level of cholesterol in the medium to lyso-phosphatidylcholine and lyso-phosphatidylethanolamine, which react with lymphocyte membranes leading to toxicity. Effects of the lyso-derivatives increase with time as more are produced, substantiated by an increase in culture toxicity noted with increased duration of exposure (3 hrs vs. 20 hrs). In an *in vivo* situation, lyso-derivatives will not be formed due to the abundance of the acceptor molecule, cholesterol, resulting in the formation of cholesterol acyl ester. (Note: The rate of catalysis for the acyl transfer to cholesterol is more than 20X higher than that of the hydrolysis of phospholipids.)

7.3.2.8 *In* vivo Mouse Micronucleus Test

A. Procedure

The objective of this study is to determine the potential of the enzyme 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 the enzyme 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.

B. 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 concluded to be a dosing/gavaging error, due to the presence of blood in the oral and nasal cavities.

The main test was performed using male mice only, because 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 enzyme groups. The positive control produced a significant increase in micronuclei PCE, substantiating the sensitivity and validity of the assay.

C. 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).

7.4 Overall Safety Assessment and Conclusions

The GCAT enzyme is not an irritant to the eyes and skin. According to the Directive of the Commission 93/21/EEC of April 27, 1993, the enzyme is non hazardous based on acute oral and irritation studies. In genotoxicity studies, the enzyme is not mutagenic, clastogenic or aneugenic. Daily oral administration of the enzyme 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.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, 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

7.4.2 Human Exposure Assessment

The GCAT enzyme provides benefits in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat.

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	
Cheese	0.27	1000	0.27	
Processed meat	0.67	300	0.20	
TOTAL for US			**1.45**	**0.039**
Danish Population				
Oils	0.83	1000	0.83	
Eggs/bakery	0.17	500	0.09	
Eggs/mayonnaise	0.02	5000	0.10	
Cheese	0.52	1000	0.52	
Processed meat	0.70	300	0.21	
TOTAL for DK			**1.75**	**0.047**

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

Estimated Daily Intake of Acyltransferase BL1

The estimated intake of this GCAT 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[19]. The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007).

The maximum estimated daily intake of the enzyme for the US and Danish population is 0.039 and 0.047 mg total protein/kg bw/day, respectively, and these estimates represent a worst case scenario.

The maximum estimated daily intake of the enzyme from degumming of oil in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat

products is 0.047 mg total protein/kg bw/day under the scenario that (1) all above commodities are treated with the enzyme, (2) 100% of the enzyme remains in the product after processing, and (3) all consumers eat all these commodities treated with the enzyme. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing (see discussion in 6.2).

Maximum Estimated Daily Intake of the GCAT enzyme = 0.047 mg/kg bw

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, in cheese and in processed meat products (i.e., cumulative risk), the NOAEL of 41 mg total protein/kg bw/day still offers an 872X fold margin of safety and the enzyme preparation is safe for these intended uses.

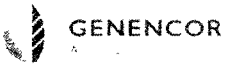
7.5 Conclusion

The safety of this GCAT enzyme 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 is assessed in a battery of toxicology studies investigating its acute oral, irritation, mutagenic and systemic toxicity potential. The enzyme is not an eye and skin irritant and is not acutely toxic. A battery of genotoxicity assays was conducted and under the conditions of these assays the enzyme is not a mutagen, not a clastogen, not an aneugen and does not increase the formation of micronuclei in bone marrow erythrocytes. Although the enzyme demonstrates cytotoxicity in *in vitro* cultures, no similar adverse effects are found in the whole animal. Daily administration of the enzyme 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).

8. BASIS FOR GENERAL RECOGNITION OF SAFETY

As noted in the Safety sections above, enzyme preparations derived from *Bacillus licheniformis*, including α-amylase, protease, pullulanase, and xylanase, are well recognized by qualified experts as being safe. Published literature, government laws and regulations, reviews by expert panels such as FAO/WHO JECFA, as well as Genencor's own unpublished safety studies, support such a conclusion.

Bacillus licheniformis is widely used by enzyme manufacturers around the world for the production of enzyme preparations for use in human food, animal feed, and numerous industrial enzyme applications. It is a known safe host for enzyme production. An enzyme preparation derived from a recombinant microorganism will be safe if the host microorganism is nonpathogenic and non-toxigenic; 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. Information is provided in this document to support that the components of the enzyme preparation meet these standards.



The safety studies conducted by Genencor established a NOAEL at 41.0 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day.

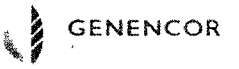
Utilizing the information provided, the enzyme preparation was taken through the Pariza-Johnson decision tree for microbial enzymes (see Appendix 4) and the preparation was determined to be acceptable for use in foods as described above.

Based on the available data from the literature and generated by Genencor, the company has concluded that the enzyme preparation from *Bacillus licheniformis* strain 3265 is safe and suitable for use in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products.

In addition, the safety determination including construction of the production organism, the production process and materials, and safety of the product were reviewed by Dr. Michael W. Pariza, Dr. Herbert Blumenthal and Dr. Joseph F. Borzelleca and they concurred with the company's conclusion that the product is GRAS (see Appendix 5). The panel was provided with:

- 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[1].

The panel independently critically evaluated these documents and other materials deemed relevant. The panel concluded that the GCAT meeting the described specifications and produced in compliance with cGMP is GRAS by scientific procedures for used in foods as described by Genencor.



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10. LIST OF APPENDICES

Appendix 1 - The amino acid sequence of GCAT

Appendix 2 - The Manufacturing process

Appendix 3 – Risk Assessment for potential food allergenicity

Appendix 4 - The Pariza/Johnson Decision Tree

Appendix 5 - GRAS Panel Report

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Appendix 1 – The amino acid sequence of GCAT

Protein sequence comparison of this GCAT to wild type *Aeromonas salmonicida* subsp. *salmonicida* GCAT (gi|39028). Only the mature parts are aligned. Residues identical in the two amino acid sequences are represented by a dot, and changes are indicated in red font.

```
Reference molecule:   gi|39028|emb|CAA50021.1|    19 -   335   (  317 aa)  Homology
       Sequence  2:   KLM3'\(mat         1 -   317   (  317 aa)     100%

Alignment type:       Local (FastScan)
Homology details:     Percent Max 99; Score 631; Length 317; Conserv N


gi|39028|emb (    19) adtrpafsrivmfgdslsdtgkmyskmrgylpsspyyegrfsngpvwleqltkqfpglt
KLM3'\(mat   (     1) ............................................................

gi|39028|emb (    79) ianeaeggatavaynkiswnpkyqvinnldyevtqflqkdsfkpddlvilwvgandylay
KLM3'\(mat   (    61) ...................d........................................

gi|39028|emb (   139) gwnteqdakrvrdaisdaanrmvlngakqillfnlpdlgqnpsarsqkvveavshvsayh
KLM3'\(mat   (   121) ............................................................

gi|39028|emb (   199) nkllinlarqlaptgmvklfeidkqfaemlrdpqnfglsdvenpcydggyvwkpfatrsv
KLM3'\(mat   (   181) ............................................................

gi|39028|emb (   259) stdrqlsafspqerlaiagnpllaqavaspmarrsasplnceqkmfwdqvhpttvvhaal
KLM3'\(mat   (   241) ............................................................

gi|39028|emb (   319) seraatfietqyeflah
KLM3'\(mat   (   301) .................
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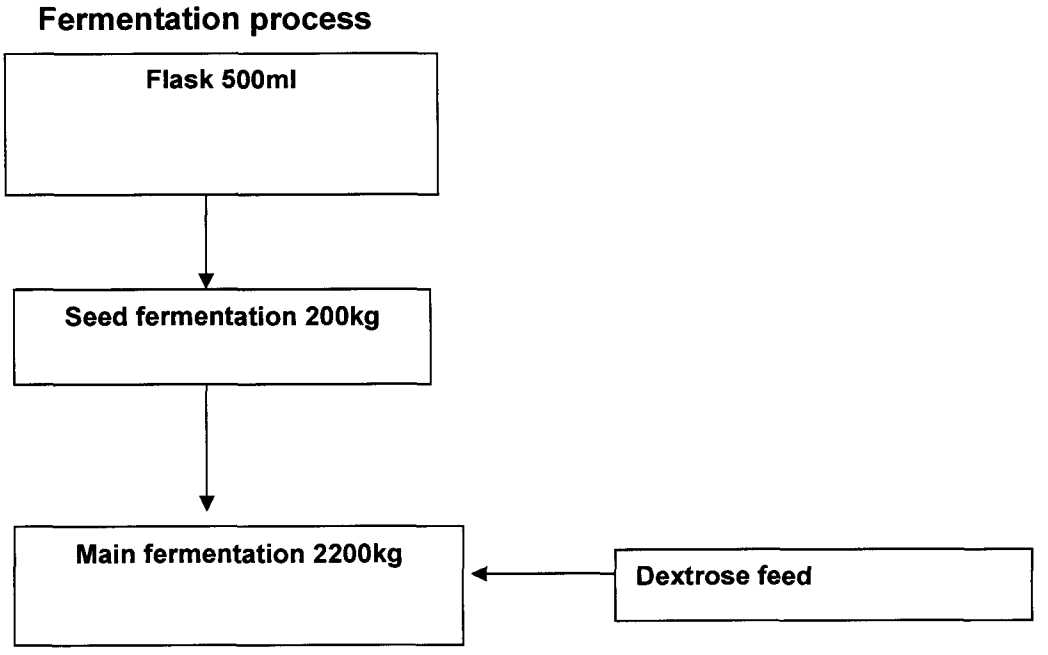
2

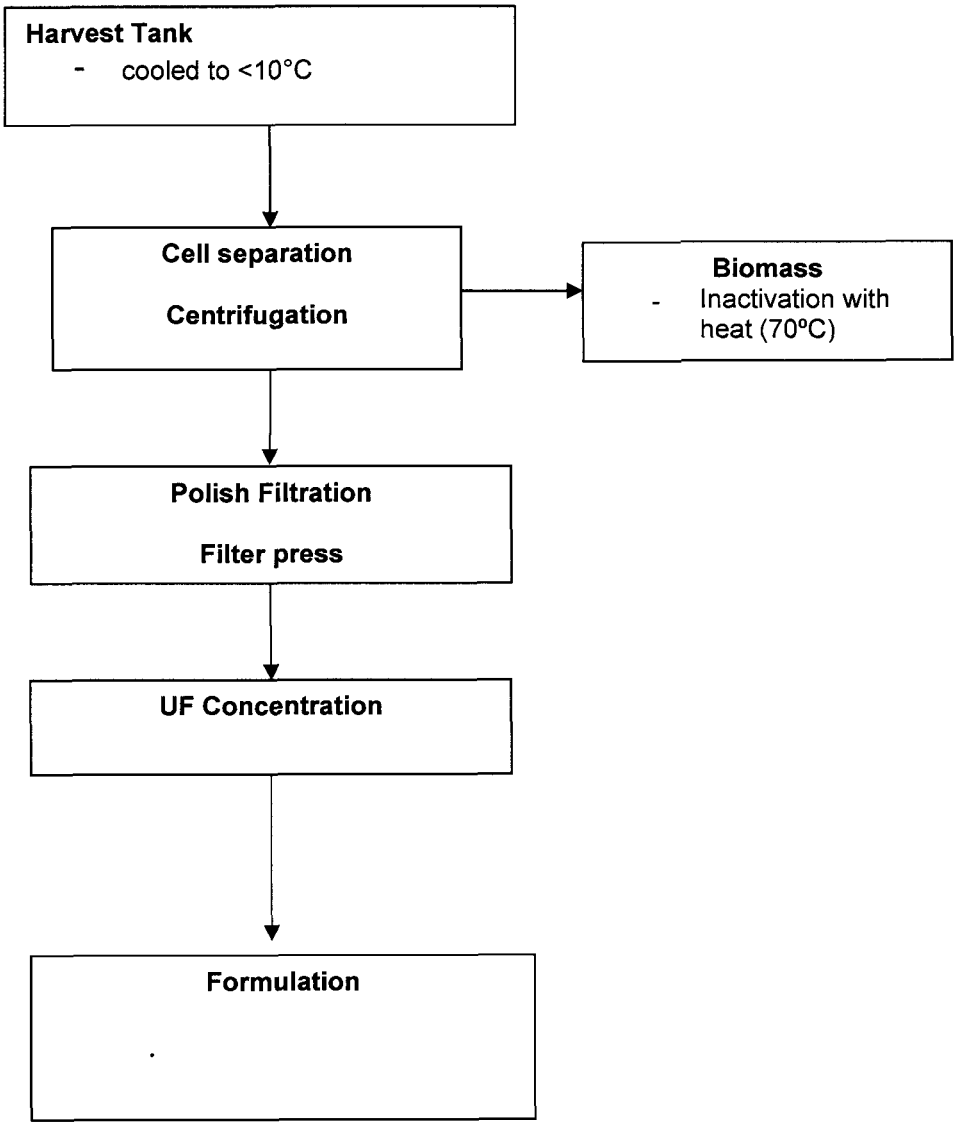
GENENCOR

Appendix 2 - The Manufacturing process



GENENCOR

Recovery flow diagram



3

Appendix 3 - Risk Assessment for potential food allergenicity

A general BLAST search using the mature GCAT 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 GCAT 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 GCAT with any allergens, using 0.01 as the maximum score to indicate homology.

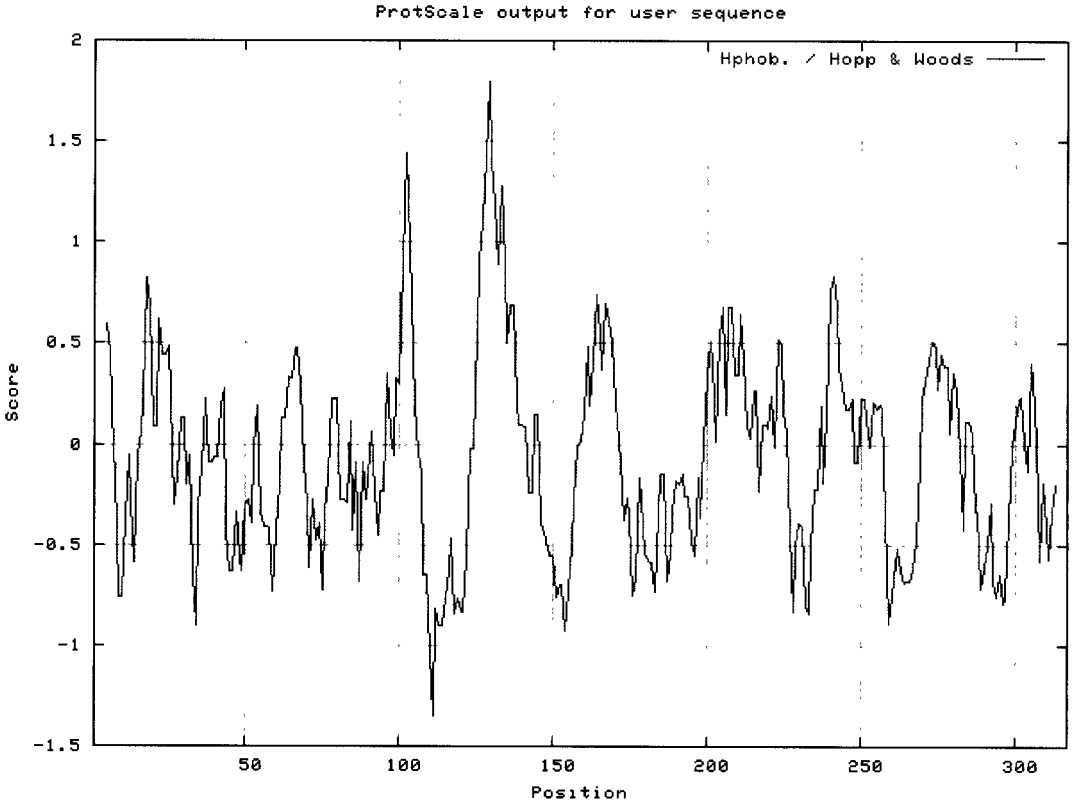
Following the recommendations in FAO/WHO (2001), two additional types of searches against the combined Allermatch database containing non-redundant allergen sequences from the SwissProt database and the WHO-IUIS list were performed using Allermatch software. The software algorithm and combined allergen 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 GCAT 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 GCAT 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), Phl 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 GCAT does not match any known food allergens.

Figure 1: Hydrophilicity profile of GCAT 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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Appendix 4 – 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

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Appendix 5 – GRAS Panel Report

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') 29 January 2008

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'.

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. A panel was assembled by Danisco USA 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.

Commercial enzyme preparations that are used in food processing 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 manufacturing process, e.g., components of the fermentation media or the residues of processing aids.

Danisco USA provided the Expert Panel with a dossier containing 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. They then spoke with representatives of Danisco USA and the Genencor Division via teleconference on December 4, 2007, 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; and
- To convert milk phospholipids to lysophospholipids, improving emulsification and increasing cheese yield by entrapping more lipid in the cheese curd.

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 *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 of 0.039 mg total protein/kg bw/day in the US and 0.047 mg total protein /kg bw/day in Denmark.

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).

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 stated that the host organism has a long history of safe industrial use and conclude 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 that the components of the enzyme preparation meet these standards.

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 studies are available from Danisco's Regulatory Affairs department in Palo Alto, CA. Danisco concludes that the results of the toxicity and mutagenicity tests demonstrate the safety of the Danisco's glycerophospholipid cholester acyltransferase KLM3' preparation and support the safe use of enzyme preparations produced by the production strain.
A NOAEL is established at 41.0 mg total protein/kg bw/day (equivalent to 116.9 mg TOS/kg bw/day), the highest dose tested.

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 in a dossier 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, areGenerally Recognized As Safe (GRAS) based on scientific procedures.

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

(b)(6) Feb. 5, 2008

Herbert Blumenthal, PhD
Consultant

(b)(6) 05 February 2008

Joseph F. Borzelleca, PhD
Professor of Pharmacology and Toxicology
Medical College of Virginia

(b)(6) 09 February 2008

Michael W. Pariza, PhD
Director, Food Research Institute
Wisconsin Distinguished Professor of Food Microbiology and Toxicology
University of Wisconsin - Madison

3-13-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Alice Caddow [alice.caddow@danisco.com]
Sent: Friday, March 13, 2009 12:51 PM
To: West-Barnette, Shayla
Subject: RE: Jury Duty

Ms. West-Barnette,

We do have efficacy data on the use of GCAT in liver sausage and treatment of egg yolks, including some data on appearance and other properties. In eggs, it will be used at the manufacturing location where the eggs are processed for use, such as at a mayonnaise plant or bakery, and not on eggs in the shell. While GCAT acts only on the egg yolk, it may be added to the whole egg in the bakery application. GCAT is used similarly to conventional phospholipases which have been used for several years without adverse organoleptic effects. We can put together a document that speaks to the points below and email it to you early next week, if that is satisfactory for you.

Thank you for your help with this matter.

Alice J. Caddow

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"West-Barnette, Shayla"
<Shayla.WestBarnette@fda.hhs.gov>

To "Alice Caddow" <alice.caddow@danisco.com>
cc
Subject RE: Jury Duty

Posted date : 03/12/2009 01:55:05 PM AST

Ms Caddow,

Thank you for taking the time to speak with us this morning. Per our conversation:

The FDA/OFAS Staff in attendance at today's meeting were:

Shayla West-Barnette
Jeanette Glew
Jannavi Srinivasan
Zofia Olempska-Beer
Stacey Williams

Following review of the amendment to GRN 265, FSIS made the following comments:

1) Under the tenets of the Federal Meat Inspection Act (FMIA), FSIS is responsible for determining the efficacy and suitability of food ingredients and additives in meat products as well as prescribing safe conditions of use. Suitability relates to the effectiveness of the additive 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. The notice failed to provide data as to GCAT suitability for use in processed meat products to improve emulsification, contribute to better consistency and reduce cooking loss.
Danisco has not provided any data which establishes that the ingredient, when used at the suggested level, accomplishes the intended technical effect. Consequently, from the standpoint of use in meat products, we would not consider this to be a complete petition until Danisco provides data which establish that the additive is being used at the lowest level necessary to achieve the intended functional effect in the specific meat and egg products to which application is desired. In addition, organoleptic data (e.g., color, taste, texture) need to be submitted showing what effects, if any, there are on products formulated with this enzyme preparation.
2) Please clarify the intended uses of GCAT in eggs (ex. whole eggs vs. egg yolks)--please note that this will be of use to both OFAS and FSIS.
So that the OFAS review team can prepare for the next step in the review of this GRN, please indicate whether the fulfillment of the FSIS requests will require no new data and will take just a few days to complete, or if further work that will take a longer period of time is necessary.
Thank you again for working with us on this matter. If you have any questions, please feel free to contact me.
Regards,
Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (office)
Shayla.WestBarnette@fda.hhs.gov

From: Alice Caddow [mailto:alice.caddow@danisco.com]
Sent: Wednesday, March 11, 2009 2:30 PM
To: Srinivasan, Jannavi; West-Barnette, Shayla
Subject: Re: Jury Duty

I am excused from jury duty so can speak with you tomorrow. Regards, Alice

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3-17-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Alice Caddow [alice.caddow@danisco.com]

Sent: Tuesday, March 17, 2009 11:35 AM

To: West-Barnette, Shayla

Subject: RE: GRN 265

Attachments: GRSN amendment 17Mar09.doc; KLM3 in liver sausages 17MAR09.pdf; Modification of egg yolk 17MAR09.pdf

Dear Dr. West-Barnette,

I have drafted an amendment to the GRAS notice which I believe responds to the questions from FSIS and OFAS. One point I need to correct from my earlier email is that the egg yolks for mayonnaise are typically treated at the egg processing plant, pasteurized and then sold as a refrigerated liquid or spray dried.

I attached the draft amendment here as a Word file so that you can put any comments you may have using the Track changes feature. Once we agree that this should be submitted, we will finalize and send in. I will be in Europe until March 27 after this evening, but will be looking at email and Dr. Vincent Sewalt of my staff is also able to assist. He can be reached at vincent.sewalt@danisco.com or 650-846-5861, if I don't respond.

Thank you again for your assistance in this matter.

Sincerely,

Alice J. Caddow

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March 17, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRSN 000265

Dear Dr. Martin:

We are writing in response to questions posed by FSIS and OFAS on the above GRAS Notice which were conveyed by phone conference with several OFAS staff, and email from Shayla West-Barnette, Ph.D., Consumer Safety Officer on March 12, 2009.

In response to the request to clarify how the enzyme will be used to treat eggs, we offer the following. The action of the enzyme will be on egg yolk. For use of the egg yolk in applications such as mayonnaise, the eggs will we treated at an egg processing plant. After being separated from the egg whites, the yolks will be treated with the enzyme and pasteurized at 80° C or greater which inactivates the enzyme. They can then be sold as treated liquid yolk that is refrigerated until use or are spray dried into powder. For the baking application, the eggs will be treated by the bakery as whole eggs, but the intended effect is only on the yolk.

We have also included two studies conducted by Genencor, one on the treatment of liver sausage with KLM3' and the other on the treatment of egg yolk. Both studies demonstrate that the enzyme is effective at emulsifying the food substrate. The enzyme treated lever sausages were tested organoleptically without any adverse effects compared with a control sausage without enzyme treatment. The structure of the enzyme treated sausages were evaluated organoleptically positive compared with the control sausage unpublished report of Danisco personnel) . The mayonnaise made using KLM3' looked similar to mayonnaise made with untreated egg yolk and was comparable in taste (unpublished report of Danisco personnel).

In the GRAS notice, we indicated that the enzyme preparation will be added to processed meats at 300LATU/kg food equal to 22.6 mg TOS/kg food and to eggs for mayonnaise production at a maximum of 5000 LATU/kg food equal to 376.9 mg TOS/kg food . In the attached study on liver sausage, the enzyme was added from 340 LATU/kg to 790 LATU/kg food equal to 25.6-59.5 mg TOS/kg food. In practice, egg yolk for mayonnaise will be treated at 2500 LATU/kg maximum equal to 188.5 mg TOS/kg food; however, 500 to 1000 LATU/g will be the normal working dosage level. In practice, the enzyme

will be added at the lowest dose needed to gain the desired technical affect. We have modified the calculation of human exposure below to take into account the maximum levels suggested from the enclosed studies.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, 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

7.4.2 Human Exposure Assessment

The GCAT enzyme provides benefits in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat.

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	2500	0.05	
Cheese	0.27	1000	0.27	
Processed meat	0.67	790	0.53	
TOTAL for US			**1.73**	**0.046**
Danish Population				
Oils	0.83	1000	0.83	
Eggs/bakery	0.17	500	0.09	
Eggs/mayonnaise	0.02	2500	0.05	
Cheese	0.52	1000	0.52	
Processed meat	0.70	790	0.55	
TOTAL for DK			**2.04**	**0.054**

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

Estimated Daily Intake of Acyltransferase BL1

GENENCOR
A Danisco Division

The estimated intake of this GCAT 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[19]. The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007).

The maximum estimated daily intake of the enzyme for the US and Danish population is 0.046 and 0.054 mg total protein/kg bw/day, respectively, and these estimates represent a worst case scenario.

The maximum estimated daily intake of the enzyme from degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products is 0.054 mg total protein/kg bw/day under the scenario that (1) all above commodities are treated with the enzyme, (2) 100% of the enzyme remains in the product after processing, and (3) all consumers eat all these commodities treated with the enzyme. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing (see discussion in 6.2).

Maximum Estimated Daily Intake of the GCAT enzyme = 0.054 mg/kg bw

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, in cheese and in processed meat products (i.e., cumulative risk), the NOAEL of 41 mg total protein/kg bw/day still offers an 759X fold margin of safety and the enzyme preparation is safe for these intended uses.

This recalculation does not alter our conclusion that the KLM3' enzyme is safe for these intended uses and is GRAS. We hope that these studies and this letter have adequately answered the questions posed, if not please contact me at email: alice.caddow@danisco.com, phone: 650-846-7557 or fax at 650-845-6505.

Sincerely,

Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures

For FSIS

KLM3' – Study of Emulsification in Liver Sausages

ABSTRACT

The effect of acyltransferase KLM3' was examined in a liver/oil emulsion, first in a model system and secondly in application trials of liver sausages with a high water and a high fat content, respectively. The activity of KLM3' was also investigated by HPTLC by measuring the presence of phosphatidylcholine (phospholipid), phosphatidylethanolamine (phospholipid) and lysophospholipids (emulsifying agent).

The model system demonstrated improved oil emulsion stability with the presence of KLM3'. At a concentration of 0.07% KLM3', the liver emulsion had an oil stability of 94% compared to 51% in the control. In the application trial, the KLM3'-treated liver sausages with high fat had a lighter colour, harder texture and improved fat retention compared to the control and CITREM-treated liver sausage. The results from HPTLC confirmed the activity of KLM3' in liver emulsion and liver sausage by degradation of phospholipids and accumulation of lysophospholipids, and formation of cholesterol ester in the enzyme-treated emulsion compared to the control. A higher dosage of KLM3' correlated to an increased activity, which seemed to have the negative effect of further degradation of the lysophospholipids in liver sausages.

INTRODUCTION

In emulsified meat products with a considerable fat content, e.g. emulsified sausages and pâtés, it is desirable to have fat stability so that fat losses are minimized and the amount of visible fat is reduced. Additionally, it is desired that the loss of meat juice is low and that the taste, texture and appearance of the product are acceptable. Emulsifiers may be added to achieve these effects and some of the most commonly known are isolated protein or protein concentrates like soy protein or Na-caseinate. However, these proteins are characterised by being relatively expensive, and the level allowed in meat products is limited. Additives like mono- and di-glycerides and citric acid esters can also be used as emulsifiers, but their application is often unwanted due to price or labelling (no additives on the label of the meat product).

Thus, alternatives to these existing emulsifiers are most welcome in the meat business. Recently, an enzymatic method was applied as an emulsifier in food products. In a study by Lilbæk et al., 2006 where mozzarella cheese was treated with a phospholipase A, they observed reduced fat losses in whey and cooking water and moisture retention in the cheese curd. In emulsified meat products it could be interesting to test such an enzymatic method with KLM3'.

The objective of the present work was to evaluate the effect of KLM3' on the yield and texture of liver sausages, and to investigate the changes in phospholipids and cholesterol levels. Liver sausages usually

have a low content of meat and a high content of non-mixable fat/water, and it is necessary to use emulsifiers to avoid separation of fat. GRINDSTED® CITREM N 12 Citric Acid Ester, which KLM3' was compared to, is a commercial product from Danisco normally used as an emulsifier in liver sausages.

TECHNOLOGY

Liver/oil emulsion

Initially, enzyme (Acyltransferase KLM3', Batch 1026295001, Activity 1128 LATU/g) was tested in a liver/oil emulsion by using a model system as described by Zorba & Kurt, 2007 with some modifications. 20g pork liver and 80g water were mixed in a Stephan cutter at 300-600rpm for 30 seconds, 1500rpm for 30 seconds and lastly 3000rpm for 3 minutes. 100g of the blend was added to a thermomixer (Vorwerk) and the enzyme was added while stirring at level 1 (100 rpm) for 15 seconds. 300ml rapeseed oil was slowly added to the thermomixer, first at level 1 for 15 seconds and then level 6 within 3 minutes.

2 x 10ml emulsion were weighed into a test tube and capped and immediately heated at 80°C in a water bath for 30 minutes. The tubes were centrifuged at 310 x g for 20 minutes and the amounts of water and oil separated were measured. Emulsion stability was calculated from the water and oil losses of the heat-treated liver emulsion using the following equations:

Emulsion stability (%): 100 – (mL of water separated x 10) + (mL of oil separated x d x 10) (d:specific gravity of oil)

Liver sausage production

Table 1: Liver sausage recipe

	Recipe	
	High water	**High fat**
Pork liver	14.8	14.8
Pork skin	14.8	29.6
Back fat	19.7	29.6
Water	49.3	24.6
Spices liver sausage	1	1
Nitrite curing salt	0.5	0.5

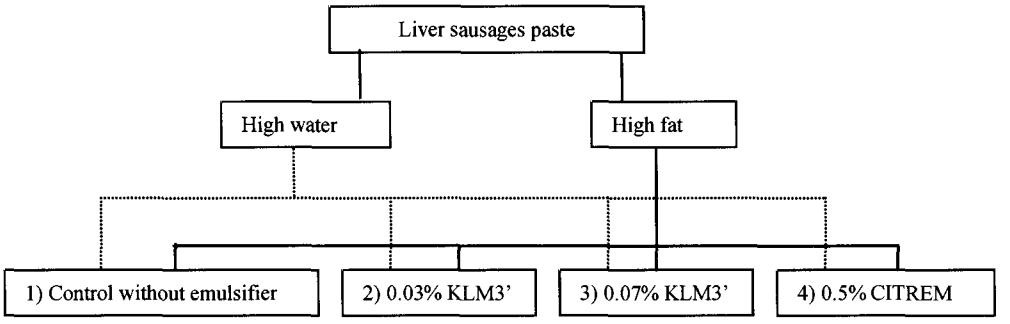


Figure 1: Experimental design of liver sausage production.

In total, 8 batches of 5kg liver sausages were produced. Half of the batches were based on a high-water recipe whereas the other half was based on a high-fat recipe (see table 1). Each of the two recipes consisted of 4 batches: 1) a control without emulsifier, 2) 0.03% KLM3', 3) 0.07% KLM3' and 4) 0.5% CITREM (figure 1).

Pork skin (3mm), back fat (3mm), hot water (80°C), spices and salt were chopped in a bowl chopper and heated with steam until a temperature of 65°C was reached. The steam was turned off and when temperature was down to 50°C, the liver was added. In recipes 2 & 3, the enzyme was added at 60°C. In recipe 4, CITREM was added at the beginning together with the spices. The chopping was stopped when 40°C was reached, and the liver paste was stuffed into tins and F-plus cal. 60 casings. Total processing time was approximately 10 minutes. The tins were autoclaved at 120°C with a 2-minute holding time, and the liver sausages were cooked at 75°C to a core temperature of 72°C.

HPTLC analysis of phospholipids

Enzyme reaction and lipid extraction:

The sample with liver paste was frozen and lyophilized. The test sample was ground in a coffee mill.
0.5g dry meat powder was extracted with 7.5ml hexane:isopropanol 3:2 for 30 minutes.
The organic phase was isolated and analysed by HPTLC.

HPTLC was used to measure the contents of free cholesterol, phospholipids and lysophospholipids in the liver paste samples.
Applicator: CAMAG applicator AST4.
HPTLC plate: 20 x 10 cm (Merck no. 1.05641)
The plate was activated before use by drying in an oven at 160°C for 20-30 minutes.

Application: 6.0µl of extracted lipids dissolved in hexane:isopropanol 3:2 was applied to the HPTLC plate using an AST4 applicator.

0.1, 0.3, 0.5, 0.8, 1.5µl of a standard solution containing standard components phosphatidylethanolamine, phosphatidylcholine and lysophospholipids) with known concentrations were also applied to the HPTLC plate

Running buffer 5: Hexane: MTBE (70:30)

Running buffer 6: Chloroform:1-propanol:Methylacetate:Methanol : 0.25% KCl in water 25:25:25:10:9

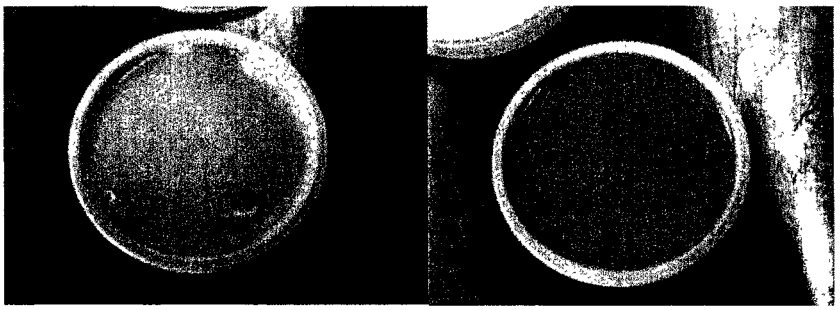
Elution length: 7cm

Developing fluid: 6% cupric acetate in 16% H_3PO_4

After elution, the plate was dried in an oven at 160°C for 10 minutes, cooled and immersed in the developing fluid (10 seconds) and then dried additionally for 6 minutes at 160°C. The plate was evaluated visually and scanned (Camag TLC scanner).

RESULTS

Model system with liver/oil emulsion



KLM3'-treated liver/oil emulsion Control liver/oil emulsion

Figure 2: Pictures of raw liver emulsion

From a visual inspection of the raw liver/oil emulsion presented in figure 2, it was observed that the control had so poor emulsion stability that it separated, whereas the KLM3'-treated liver/oil emulsion had a nice, homogeneous emulsion. Also a much lighter colour was observed in the enzyme-treated liver/oil emulsion compared to the control, which demonstrated a better emulsion.

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Table 2: Oil stability in liver/oil emulsion added KLM3' and a control without enzyme

	Control	0.03% KLM3'	0.07% KLM3'
Oil stability	51%	90%	94%

The specific gravity of the liver/oil emulsion was determined to 900kg · m^{-3} at 4°C at a normal pressure of 1 atm.

The results show that the emulsion stability is improved with the presence of KLM3'. At a concentration of 0.07% KLM3', the liver/oil emulsion had an oil stability of 94% compared to 51% in the control.

Application trial on liver sausages

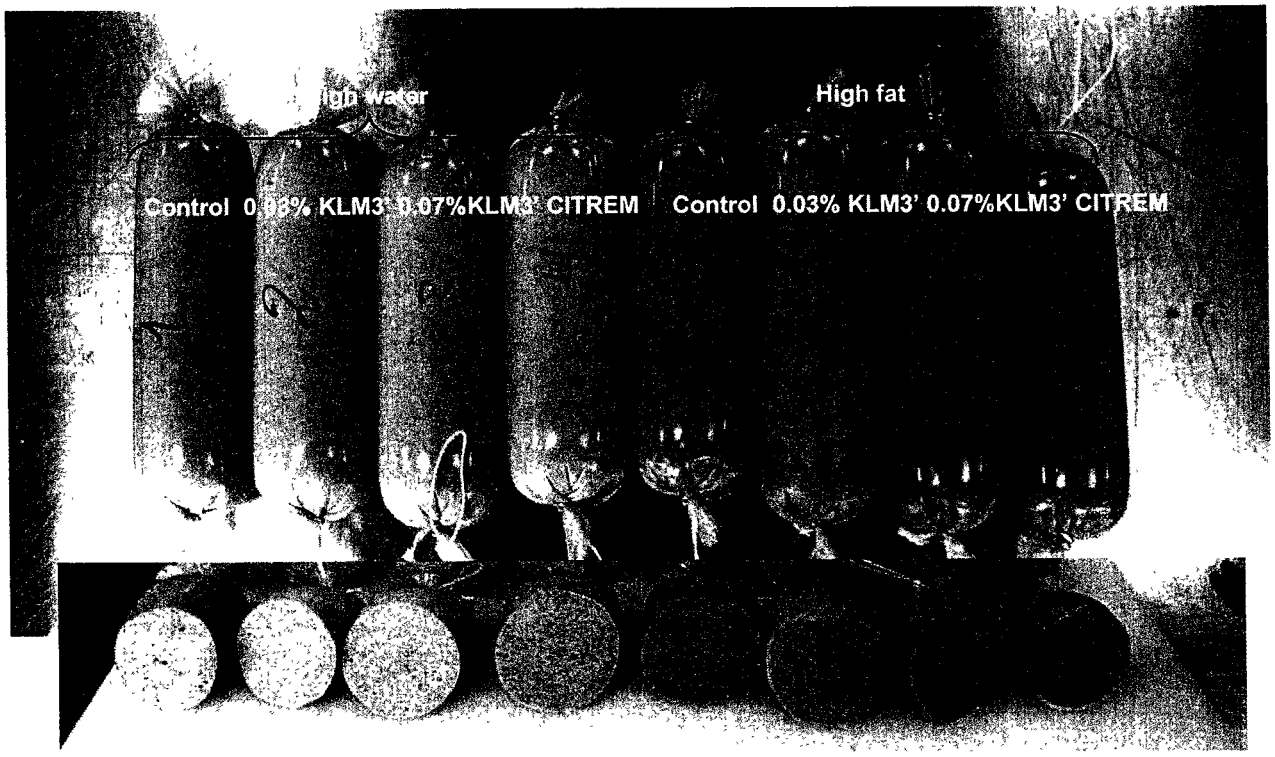

Figure 3: Pictures of heat-treated liver sausages

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A difference among the samples was first observed during processing where the enzyme-treated meat batter had a thicker consistency compared to the control and the batch with CITREM N 12.

As shown in figure 3, the colour of the sausages varied from batch to batch. In batches 1 to 4 which contained high water in the recipe, all the colours were light and it seemed that sample 4, the one with CITREM, was a bit lighter that the others. As mentioned previously, a lighter colour may indicate a better emulsion.

In the liver sausages with a high amount of fat (5-8), it seemed that a better emulsion was obtained in both enzyme-treated sausages (6 and 7); they were clearly lighter in colour compared to both the control (5) and the CITREM (8) batch. The colour of the liver sausage treated with CITREM was very dark, and a heavy separation of fat appeared on the surface.

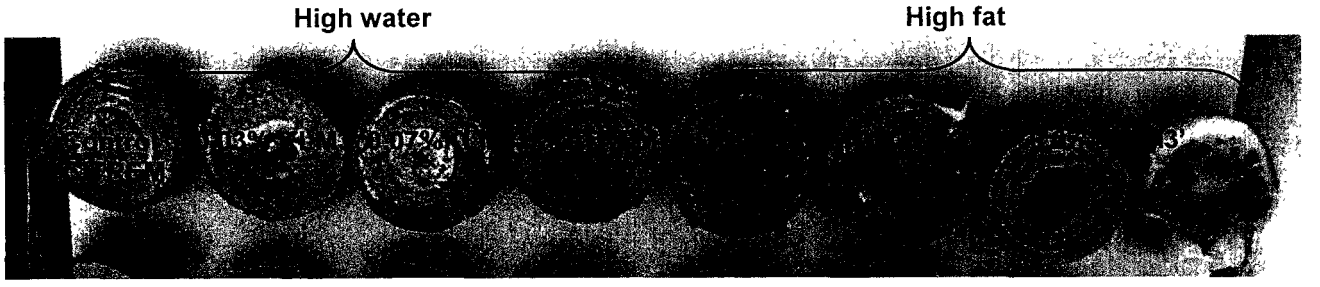

Figure 4: Picture of pasteurised liver pastes

The autoclaved samples of liver paste shown in figure 4 very well illustrate the fat and jelly separation. The samples of liver paste with a high content of water had jelly separation to a similar extent. However, there was a clear difference among the samples of liver paste with a high content of fat in which the fat separation was an indicator of poor emulsion stability. The sample with CITREM had an extreme fat separation compared to the samples treated with enzyme. Also, but to a much lower extent, the control showed some fat separation.

It seemed from the results presented that the formed lysophospholipids exhibit a strong lipophilic group within the molecule as it worked very well in an emulsion with a high fat content. On the other hand, the emulsifier CITREM had a negative impact on the liver sausages, especially the ones with a high content of fat. Most probably the content of fat in the recipe exceeded the emulsion capability of CITREM.

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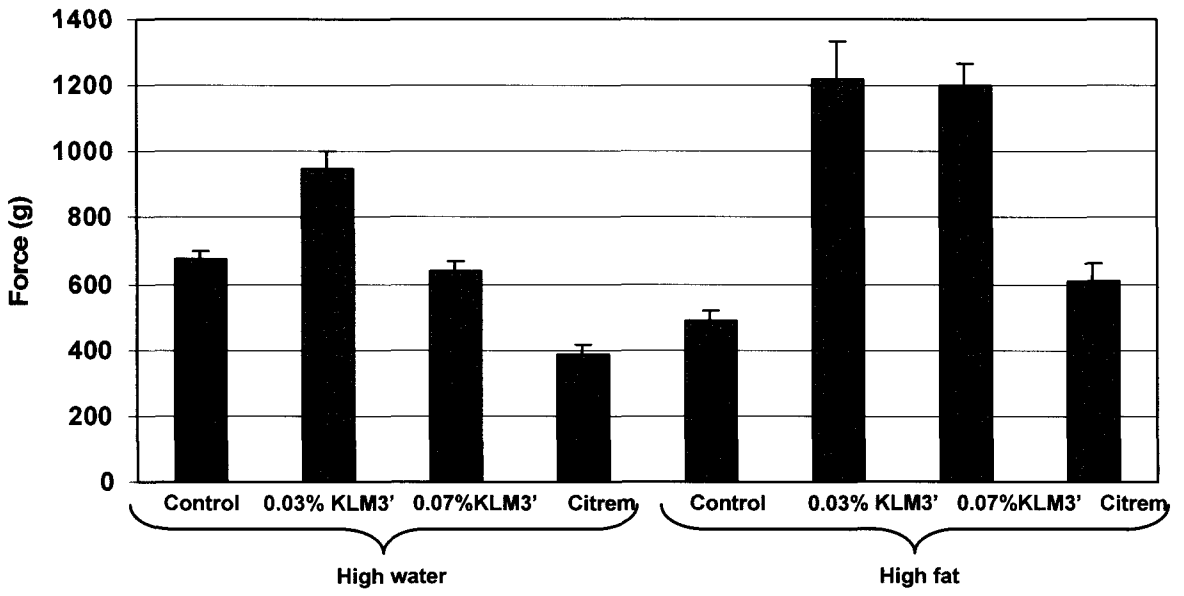

Figure 5: Texture analysis of cooked liver sausages.

From the results presented in figure 5, it seemed that the liver sausages treated with enzyme compared to the control and the CITREM-treated sausages, except from 0.07% KLM3' in a high water recipe, had a harder texture, which may be correlated to a better emulsion Note: The force needed to penetrate the sausage is a measure for the stability of the sausage emulsion. Higher force will give a better and more firm bite ,which is preferred when you eat the sausage.

HPTLC analysis

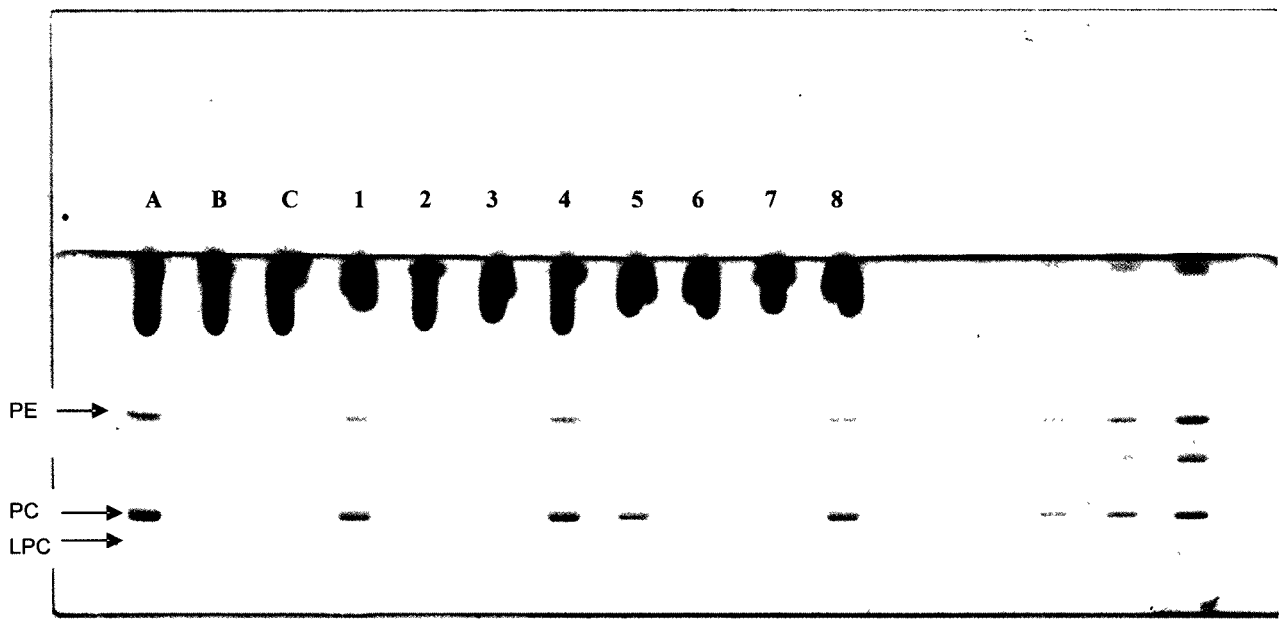

Figure 6: HPTLC analysis (running buffer 6) of phospholipids (PC) and (PE) and lysophospholipids (LPC) of liver emulsion with A) Control B) 0.03% KLM3' C) 0.067% KLM3' & of cooked low -fat liver sausages with 1) Control 2) 0.03% KLM3' 3) 0.07% KLM3' 4) CITREM and high-fat liver sausage 5) Control 6) 0.03% KLM3' 7) 0.07 %KLM3' 8) CITREM

The results from figure 6 confirm the activity of KLM3' in liver emulsions and liver sausages.

The activity of KLM3' caused a larger accumulation of lysophospholipids (LPC) in enzyme- treated emulsions B & C compared to the control A, illustrated by the appearance of LPC bands. The HPTLC analysis of liver sausages showed vague bands of lysophospholipids at both enzyme concentrations which could indicate an overdose of the enzyme in such a way that the lysophospholipids were further degraded. The bands of phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were quantified:

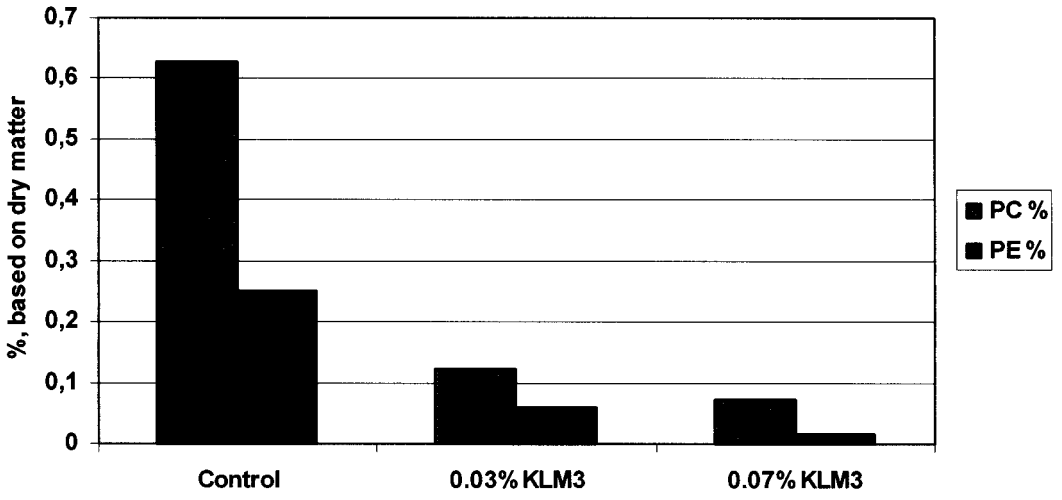

Figure 7: HPTLC analysis (running buffer 6) of phospholipids (PC) and (PE) from cooked liver/oil emulsion.

From the quantification of the primary bands presented in figure 7 it was shown that the activity of KLM3' on phospholipids caused a degradation of phospholipids PC and PE which correlated to increased concentration of enzyme in the liver/oil emulsion.

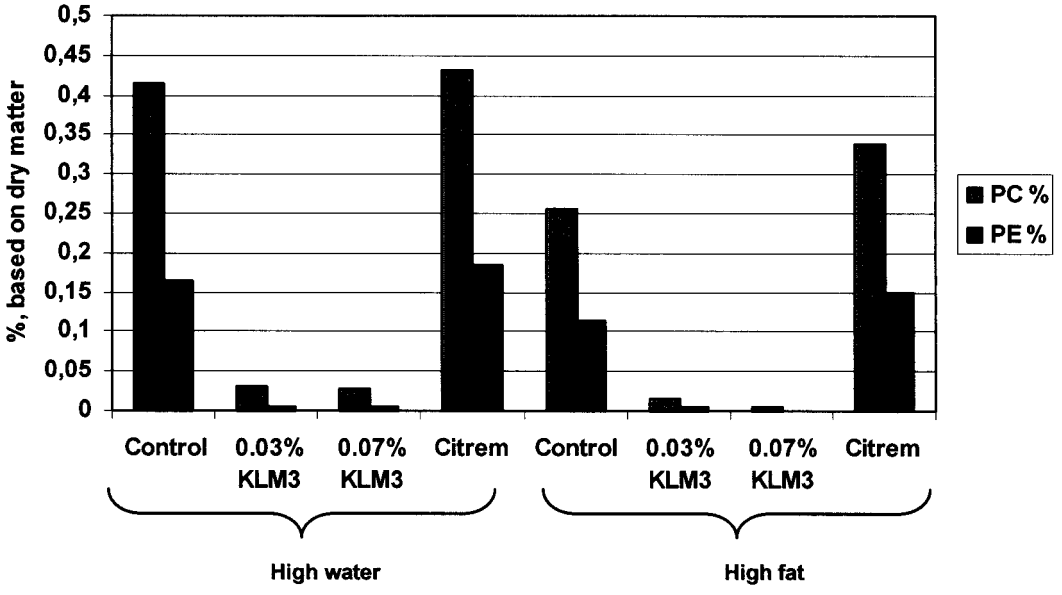

Figure 8: HPTLC analysis (running buffer 6) of phospholipids (PC) and (PE) in liver sausages

From the quantification of the primary bands presented in figure 7 it was shown that the activity of KLM3' on phospholipids caused a degradation of phospholipids, PC and PE, which correlated to increased enzyme concentration in the liver sausages.

Table 3: HPTLC analysis (running buffer 5) of cholesterol in liver sausage samples. % based on dry weight

	Control	0,03% KLM3'	Citrem N12
% Cholesterol	0.277	0.067	0.264
% cholesterol reduction	0	76	5

From a previous experiment with liver sausages/high water recipe, the effect of KLM3' on cholesterol was examined. The results in table 3 show that cholesterol was significantly reduced in the KLM3' treated liver sausages compared to the control and citrem treated sausages. This observation confirms acyltransferases ability as a converter of cholesterol.

CONCLUSION

- Results from the model system show that emulsion stability is improved with the presence of KLM3'. At a concentration of 0.07% KLM3', the liver emulsion had an oil stability of 94% compared to 51% in the control.
- The KLM3'-treated liver sausages with a high fat content had a lighter colour and improved fat retention compared to the control and CITREM-treated liver sausages. It seems that KLM3' works very well in high-fat recipes.
- Texture was much harder in the KLM3'-treated sausages with a high fat content compared to the control and the liver sausages with CITREM. To some extent, high water and 0.03% KLM3' also resulted in texture hardening.
- The results from HPTLC confirmed the activity of KLM3' in liver emulsions and liver sausages by an accumulation of lysophospholipids, and a degradation of phospholipids and conversion of free cholesterol to cholesterol ester in enzyme-treated emulsions compared to the control. A higher dosage of KLM3' correlated to an increased activity which may have the negative effect by further degradation of the lysophospholipids.

REFERENCES

Zorba, Ö. and Kurt, S., 2007: The effects of plant oils on meat emulsions. International Journal of Food Science and Technology, 1-8

Lilbæk, H. M., Broe, M. L., Høier, E., Fatum, T. M., Ipsen, R. and Sørensen, N.K., 2006: Improving the yield of Mozzaella cheese by phospholipase. J. dairy Sci. 89, 4114-4125.

KLM3' – Modification of Egg Yolk

ABSTRACT

Egg yolk contains 31% lipid, 30% of which is lecithin. Enzymatic conversion of lecithin to lyso-lecithin improves the emulsification properties of the egg yolk. When making a.o. mayonnaise these improved emulsification properties are important in preventing separation of the product upon pasteurisation. Opposed to applying conventional phospholipases for modification of egg yolk KLM3', a glycerophospholipid cholesterol acyltransferase should give a lower level of free fatty acid due to the formation of cholesterol ester and hence a mayonnaise less prone to oxidation.

An in-house ^{31}P-NMR method was set up to monitor the lecithin, lyso-lecithin level in enzymatically modified egg yolk. Egg yolk was modified with KLM3' at the following parameters: pH (5.0 -10.0), enzyme dosage (0.5-5.0 LATU/g egg yolk), incubation temperatures (50-60°C), and incubation times (15-360 min). Kinetics were followed by analysis of amount of free fatty acid and cholesterol as well as by ^{31}P-NMR analysis of the lecithin and lyso-lecithin level. The lyso-phosphatidylcholine (LPC) activity of KLM3' caused accumulated LPC to be degraded resulting in a low phosphatidylcholine (PC) conversion degree and contemporary elevated free fatty acid (FFA) levels.

Pilot scale mayonnaises made with KLM3'modified egg yolk were on level with pilot scale mayonnaises made with egg yolk modified with phospholipases, Lysomax PLA2 (Genencor) or Lipomod 699L (Biocatalyst) and with mayonnaise made with HSEYP (Heat stable egg yolk powder, Sanovo) with respect to viscosity, particle size distribution and heat stability despite the lower PC conversion ratio for KLM3' (50% versus 75-95 %). Opposed to expected, mayonnaises made with KLM3' modified egg yolk did not show improved oxidation stability compared with mayonnaises made with Lipomod 699L or Lysomax modified egg yolk or with HSEYP, due to the elevated FFA level in KLM3' modified egg yolk caused by the LPC activity.

The trials made with pilot scale mayonnaises showed that the egg content can be reduced by 50% (from 5% to 2.5%) when using enzyme modified egg yolk instead of unmodified egg yolk, irrespective of the enzyme used for the modification.

ABRIVIATIONS

FFA: Free fatty Acid
GPC: Glycerophospholipd
HSEYP: Heat Stable Egg Yolk Powder
LPC: Lyso-phosphatidylcholine
LPE: Lyso-phosphatidylethanolamine
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
TPP: Triphenylphosphate

INTRODUCTION

Egg yolk is well known for use in the food industry due to its emulsification properties. Approximately 31% of egg yolk is lipid and 31% of the lipid is lecithin, see Figure 1. Lecithin contributes to egg yolk's 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, pasteurisation of the product at high temperatures causes the product to separate. Changing the lecithin:lyso-lecithin ratio will improve the emulsifying properties of egg yolk because lyso-phospholipid is a better oil in water emulsifier than lecithin. Improved emulsifying properties of egg yolks are of importance when making a.o. heat stable mayonnaise

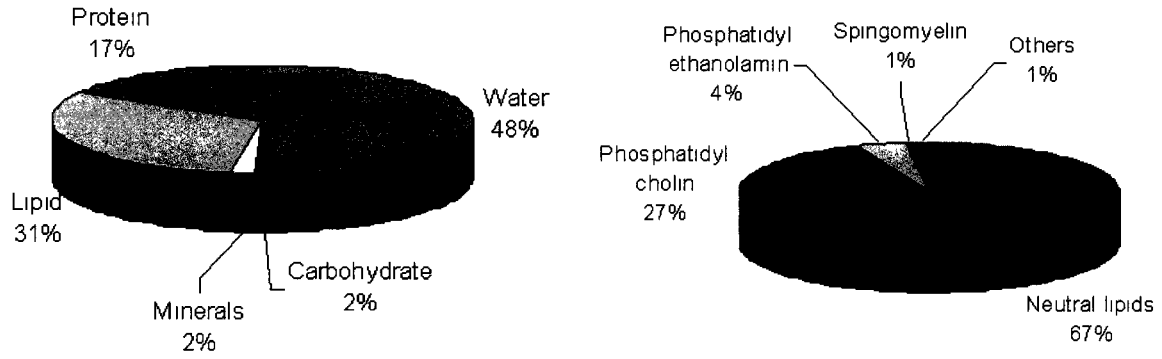

Figure 1: Egg yolk composition, and lipid distribution (www.aeg.org)

The acyltransferase KLM3' catalyses the conversion of the phospholipid and cholesterol to lyso-phospholipid and cholesterolester in egg yolk, see Figure 2.

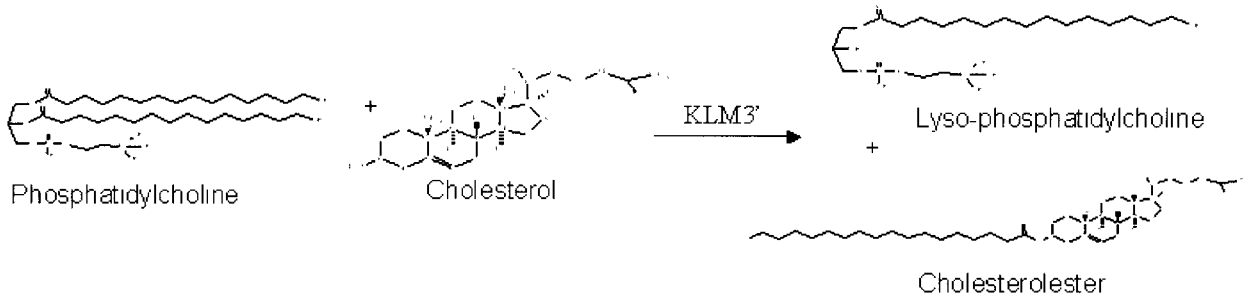

Figure 2: The acyltransferase KLM3' catalyses the conversion of phosphatidylcholine and cholesterol to lyso-phospahatidylcholine and cholesterolester, respectively.

The aim of this study was to examine the conversion of phospholipid in egg yolk based on a kinetic study with KLM3' in egg yolk. Effects of incubation time, temperature, and pH as well as the effect of KLM3' enzyme dosage were investigated. Furthermore, pilot scale mayonnaise production with KLM3' modified egg yolk was performed.

The end product was evaluated with respect to the following characteristics: viscosity, particle size distribution, heat stability, and oxidation stability. References used were Lysomax PLA2, Genencor and Lipomod 699L, Biocatalyst treated egg yolk as well as Heat Stable Egg Yolk Powder (HSEYP), Sanovo A/S, and untreated egg yolk.

MATERIALS AND METHODS

Enzyme:
K460: KLM3' acyltransferase from *Aeromonas salmonicida* expressed in *Bacillus licheniformis* from fermentation DW3206-49, formulated on 35% glycerol and 0.5% potassium sorbate at 1505 LATU/ml. Diluted to 150 LATU/ml in demineralised water before use.
#3395, FoodPro LysoMax PLA2, Genencor, lot 102-06067-001
#3332, Lipomod 699L, Biocatalyst, lot 10510125

Enzymatic treatment, kinetic study:

pH: Enzymatc treatment with a dosage of 2.0 LATU/g yolk at 50°C for 120 minutes was carried out at the following pH values: 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, and 10.0. Egg yolk has a natural pH value of 6.0. Initial pH adjustment of pasteurised egg yolk with 8% NaCl from Sanovo A/S, Denmark, to the values listed above was made with citric acid or NaOH. 15 g of the pH adjusted egg yolk was transferred to a Wheaton tube and placed in a heating block controlled to 50°C. At time t=0, KLM3' enzyme stock solution was added to the egg yolk according to Table 1 to obtain an enzyme dosage of 2.0 LATU/g yolk and a control was included as well. At time t=120 minutes, 1.0 g samples were taken from the egg yolk/enzyme solutions. The enzymatic reaction in the samples was stopped by adding 7.5 ml organic solvent ($CHCl_3$:MeOH (2:1 (v/v)).

Temperature: Enzymatic treatment was carried out at the three following temperatures: 50°C, 55°C, and 60°C, each with the four following dosages: 1.5 LATU/g yolk, 2.0 LATU/g yolk, 3.0 LATU/g yolk, and 5.0 LATU/g yolk. In addition, at 55°C the three following dosages were tested: 0.5 LATU/g yolk, 1.0 LATU/g yolk, and 2.5 LATU/g yolk. At 60°C the dosage of 2.5 LATU/g yolk was also tested.

15 g pasteurised egg yolk with 8% NaCl from Sanovo A/S, Denmark, was transferred to a Wheaton tube and placed in a heating block controlled to the appropriate temperature. At time t=0, KLM3' enzyme stock solution was added to the egg yolk according to Table 1 to obtain the different enzyme dosages and a control was included as well. At time t=15 minutes, 30 minutes, 60 minutes, 120 minutes, 180 minutes, 240 minutes, and 360 minutes, 1.0 g samples were taken from the egg yolk/enzyme solutions. The enzymatic reaction in the samples was stopped by adding 7.5 ml organic solvent ($CHCl_3$:MeOH, (2:1 (v/v))).

Table 1: Amount of KLM3' enzyme stock solution (150 LATU/g yolk) added to egg yolk to obtain different enzyme dosages and a control was included as well. Demineralised water was added to 0.5 ml to eliminate any difference in volume upon adding different volumes of enzyme stock solution.

Sample	Amount egg yolk	Enzyme activity	Volume enzyme stock solution added	Volume dem. H_2O added
Control	15.0 g	0 LATU	0 ml	0.5 ml
0.5 LATU/g yolk	15.0 g	7.5 LATU	0.05 ml	0.45 ml
1.0 LATU/g yolk	15.0 g	15.0 LATU	0.10 ml	0.40 ml
1.5 LATU/g yolk	15.0 g	22.5 LATU	0.15 ml	0.35 ml
2.0 LATU/g yolk	15.0 g	30.0 LATU	0.20 ml	0.30 ml
2.5 LATU/g yolk	15.0 g	37.5 LATU	0.25 ml	0.25 ml
3.0 LATU/g yolk	15.0 g	45.0 LATU	0.30 ml	0.20 ml
5.0 LATU/g yolk	15.0 g	75.0 LATU	0.50 ml	0 ml

Lipid extraction:
Addition of 7.5 ml organic solvent ($CHCl_3$:MeOH, (2:1 (v/v))) to the sample did not only stop the enzyme reaction, but also extracted the lipids. Furthermore, 0.2 ml demineralised H_2O was added to the sample before it was dispersed using a Whirley mixer for 1 minute. The sample was then centrifuged for 10 minutes at 110 x g. Approximately 3 ml of the organic phase was transferred to a different tube and this extracted lipid was used for various analyses.

Free fatty acid analysis:
100 µl of the extracted lipid solution was evaporated under nitrogen at 50°C. 1.0 ml demineralised H_2O was added and the lipid was dispersed using a Whirley mixer. The amount of free fatty acid was determined on Konelab Autoanalyser (Thermo, Finland) using the NEFA C kit (WAKO GmbH, Germany). Assay was run at 37°C. 75 µl solution A and 15 µl redispersed extracted lipids were incubated for 10 minutes. 150 µl solution B was added and incubated for 10 minutes. The absorbance at 520 nm was measured. The amount of free fatty acid was determined, using the read absorbance and a standard curve based on oleic acid (0.05 mM to 1.0 mM).

Cholesterol analysis:
100 µl of the extracted lipid solution was evaporated under nitrogen at 50°C. 1.0 ml EtOH was added and the cholesterol was dispersed using a Whirley mixer. Analysis was carried out using a Konelab Autoanalyzer (Thermo, Finland) and a coupled enzymatic assay applying cholesterol oxidase and ABTS and peroxidase. The assay was run at 37°C. 100 µl substrate (1.25 U/ml cholesterol oxidase, 0.04% ABTS, 0.05% Peroxidase in 100 mM Tris/HCL, pH 6.6, 0.45% Triton) was incubated for ten minutes before 5µl redispersed extracted lipid solution was added. After five minutes incubation, OD 405 nm was read. The amount of cholesterol was determined using the read absorbance and a standard curve based on cholesterol (0.05 mg/ml to 0.40 mg/ml).

^{31}P NMR analysis:
NMR-SAMPLE PREPARATION:
Preparation of CsEDTA-solution: 0.2 M EDTA in MilliporeQ-water was titrated with 0.2M CsOH x1H_2O to a pH=10.5.
IS-solution: 250mg Triphenylphosphate (TPP) in 5 ml CDCl3/MeOH (2:1 (v/v))
2.0 ml of the extracted lipid solution was evaporated and re-dissolved in 2.0 ml $CDCl_3$/MeOH (2:1 (v/v)), washed with 2.0 ml CsEDTA(aq) and adjusted to pH=10.5. After centrifugation (4500rpm/5mins), 800 µl of the lower phase was placed in a 5 mm NMR-tube and 5mm NMR-tube and 25µl of the IS-solution (1.25mg TPP) was added.

NMR-CONDITIONS:
Spectra were recorded using a Varian Mercury 200vx (200MHz – ^{1}H resonance frequency) NMR-instrument at 81MHz (^{31}P resonance frequency) equipped with a liquid BB-probe. Parameters used: full proton decoupling, spectral width=2500Hz, temperature=28°C, pw=9.8μsec/52dB, d1= 10sec., number of scans =256.

Chemical shifts (PC at δ=-0.87ppm, 2-LPC at δ=-0.42ppm, 1-LPC at δ=-0.54ppm, PE at δ=-0.10ppm and LPE at δ=0.24ppm) were relative to an external standard (H_3PO_4 at δ=0.0ppm and checked using an internal standard TPP at δ=-17.84ppm).

Data were processed and integrated in Varian VNMR vers.1.6a (lb=1.0) and MestRe-C vers.4.9.9.6.

Enzymatic treatment, yolk for mayonnaise:

For the production of mayonnaise, pasteurized egg yolk with 8% NaCl from Sanovo A/S, Denmark, was enzymatically treated according to Table 2. Enzymatic treatment was carried out with slow agitation in either a heating block or a water bath at appropriate temperatures. To simulate pasteurization after treatment, the egg yolks were heated to 68-72°C with a holding time of 3 minutes applying a water bath at 85°C. Samples were taken and treated as under Lipid extraction.

Table 2: Enzymatic treatment of egg yolk from Sanovo A/S using KLM3', Lysomax PLA2 and Lipomod 699L, respectively. The KLM3' solution used had an activity of 150 LATU/ml, the Lysomax PLA2 had an activity of 473 U/ml, and the Lipomod 699L had an activity of 2000 U/ml.

Sample	**KLM3'-K460 Dosage/g yolk**	**Lysomax PLA2 Dosage/g yolk**	**Lipomod 699L Dosage/g yolk**	**Temperature (°C)**	**Time (min)**
2344-89-Control-60#	-	-	-	60	240
2344-89-K460#	2.0 LATU	-	-	60	240
2344-89-Lysomax#	-	5 U		45	180
2344-89-Lipomod#	-	-	15 U	45	180
2344-93-K460-60*	1.5 LATU	-	-	55	60
2344-93-K460-240*	1.5 LATU	-	-	55	240
2344-93-Lipomod*	-	-	15 U	45	180
2344-93-Control-45*	-	-	-	45	180
2344-94-K460#	1.5 LATU	-	-	50	20
2344-94-Lipomod#	-	-	15 U	45	180

#Enzymatic treatment in heating block.
*Enzymatic treatment in water bath.

Production of mayonnaise:

Mayonnaise with varying contents of egg yolk was produced using a FrymaKoruma mixer (Disho A15) (Table 3). Water, salt, sugar and potassium sorbate were mixed. For mayonnaises made with stabilizer, Grinsted FF 5105 was mixed 2:1 with oil before addition to the water phase. When all ingredients were dissolved, the egg yolk was added. Under vacuum, the oil was then emulsified into the water phase (stirring speed 3500 rph). Finally, vinegar and mustard were added.

Table 3: Ingredients used to produce mayonnaise with stabiliser.

Ingredient	**Mayonnaise 5% yolk**	**Mayonnaise 3% yolk**	**Mayonnaise 2.5% yolk**	**Mayonnaise 1.5 % yolk**	**Mayonnaise 2+1 % yolk***
Water	9.5 %	11.5 %	12 %	13.0 %	34.5 %
Oil	80.0 %	80.0 %	80.0 %	80.0 %	50.0 %
Modified yolk	5.0 %	3.0 %	2.5 %	1.5 %	2.0 %
Raw yolk	-	-	-	-	1.0 %
Salt	0.7 %	0.7 %	0.7 %	0.7 %	1.0 %
Sugar	1.0 %	1.0 %	1.0 %	1.0 %	3.0 %
Potassium Sorbate	0.1 %	0.1 %	0.1 %	0.1 %	0.1 %
Grindsted FF 5105	0.2 %	0.2 %	0.2 %	0.2 %	1.7 %
Vinegar 10%	3.00 %	3.00 %	3.00 %	3.00 %	4.00 %
Mustard	0.50 %	0.50 %	0.50 %	0.50 %	1.50 %
Sum	**100 %**	**100 %**	**100 %**	**100 %**	**100 %**

*2% modified egg yolk + 1% raw egg yolk used to give a total of 3.0% yolk

Table 4: Ingredients used to produce mayonnaise without stabiliser.

Ingredient	**Mayonnaise 3% yolk**	**Mayonnaise 1.5 % yolk**
Water	11.7 %	13.2 %
Oil	80.0 %	80.0 %
Modified yolk	3.0 %	1.5 %
Raw yolk	-	-
Salt	0.7 %	0.7 %
Sugar	1.0 %	1.0 %
Potassium Sorbate	0.1 %	0.1 %
Grindsted FF 5105	-	-
Vinegar 10%	3.00 %	3.00 %
Mustard	0.50 %	0.50 %
Sum	**100 %**	**100 %**

Egg yolks modified as described under Enzymatic treatment, yolk for mayonnaise, were used to produce the mayonnaises listed in Table 5.

Table 5: Pilot scale mayonnaise produced with enzymatically modified egg yolk listed in Table 2. Red box outline indicates mayonnaise made without stabilizer.

Egg yolk used	Mayo 5% yolk	Mayo 3% yolk	Mayo 2.5% yolk	Mayo 2.0% yolk	Mayo 1.5 % yolk	Mayo 2+1 % yolk
2344-89-Control-60			FM06-31-2			
2344-89-K460			FM06-31-4			
2344-89-Lysomax			FM06-31-6			
2344-89-Lipomod			FM06-31-8			
2344-93-K460-60					FM06-31-14	
2344-93-K460-240					FM06-31-16	
2344-93-Lipomod					FM06-31-18	
2344-93-Control-45					FM06-31-20	
2344-94-K460					FM06-31-40	
2344-94-Lipomod					FM06-31-43	FM06-31-45
HSEYP, Sanovo			FM06-31-10		FM06-31-46	
			FM06-31-33	FM06-31-32	FM06-31-22/34	
Raw yolk, Sanovo						FM06-31-45

Shake test

An accelerated stability study was performed as a shake test. Mayonnaise was shaken at 300 rpm at room temperature for 72 hours.

Heat stability

To evaluate the heat stability of the mayonnaise, it was placed in a 90°C water bath for an hour. Afterwards, the degree of oiling-out was judged visually.

Viscosity measurement

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Viscosity of the mayonnaise was measured on a Brookfield, DV-II, with a Helipat spindle type T-D at 2 rpm. See procedure A100.

Oxidation stability

The mayonnaise is stressed oxidatively by heating. The breakdown of the product will induce oxygen consumption, which is registered as change in pressure by a pressure transducer. The induction period (hour), which is a measure of the oxidation stability of the product, is the time that elapses before a change in pressure is observed.

Using a ML OXIPRES (MicroLab, Århus, Denmark), a 5.2 bar oxygen pressure was applied to 20 g mayonnaise. Analysis temperature was 100°C. Induction period was read as the time that elapsed from start to the intercept between the two tangents.

Particle size determination in mayonnaise

Particle size distribution can be determined by exploiting that laser light is spread in different angels depending on the size of the particle. Small particles spread laser light in large angels, whereas large particles spread lights in small angels. The particle size distribution is based on volume, but results are expressed in particle diameters. 2.0 g mayonnaise sample was dissolved in 10 ml demineralised water. The particle size distribution was then measured on a Malvern Mastersizer S.

RESULTS AND DISCUSSION

The temperatures and pH were chosen based on the temperature and pH optima and stability anticipated. KLM3' showed optimum activity at 50-65°C and pH 7.0-10.0. Optimum stability of KLM3' was observed at temperatures of 50°C or below and at pH between 6.0 and 10.0. The enzyme dosages chosen were based on initial experiments. In Table 6, the seven pH values, the three temperatures, and the seven enzyme dosages included in the kinetic study are listed.

Table 6: Parameters evaluated in the kinetic study with KLM3' in egg yolk.

Parameter	Values
pH	5.0, 5.5, 6.0, 7.0, 8.0, 9.0 and 10.0
Temperature	50, 55 and 60°C
Enzyme dosage	0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 5.0 LATU/g yolk
Incubation time	0, 15, 30, 60, 120, 180, 240 and 360 minutes

^{31}P NMR was used to follow the change in phospholipid distribution in egg yolk as enzymatic treatment progressed over time. The molar percentage of each type of phospholipid present (Phosphatidylcholine (PC), phosphatidylethanolamine (PE), 1-Lyso-phosphatidylcholine (1-LPC), 2-Lyso-phosphatidylcholine (2-LPC), and Lyso-phosphatidylethanolamine (LPE)), as well as the internal standard Triphenylphosphate (TPP) was calculated based on integration of raw data, see Figure 3, Figure 4, Figure 5 and Table 7. Both a full NMR spectrum and a zoomed spectrum are needed to integrate the amount of TPP and the small peak of 1-LPC. The correction of the TPP integral ($TPP^* = TPP_{raw}/PC_{raw} * PC$) is performed to match PC in the zoomed spectrum.

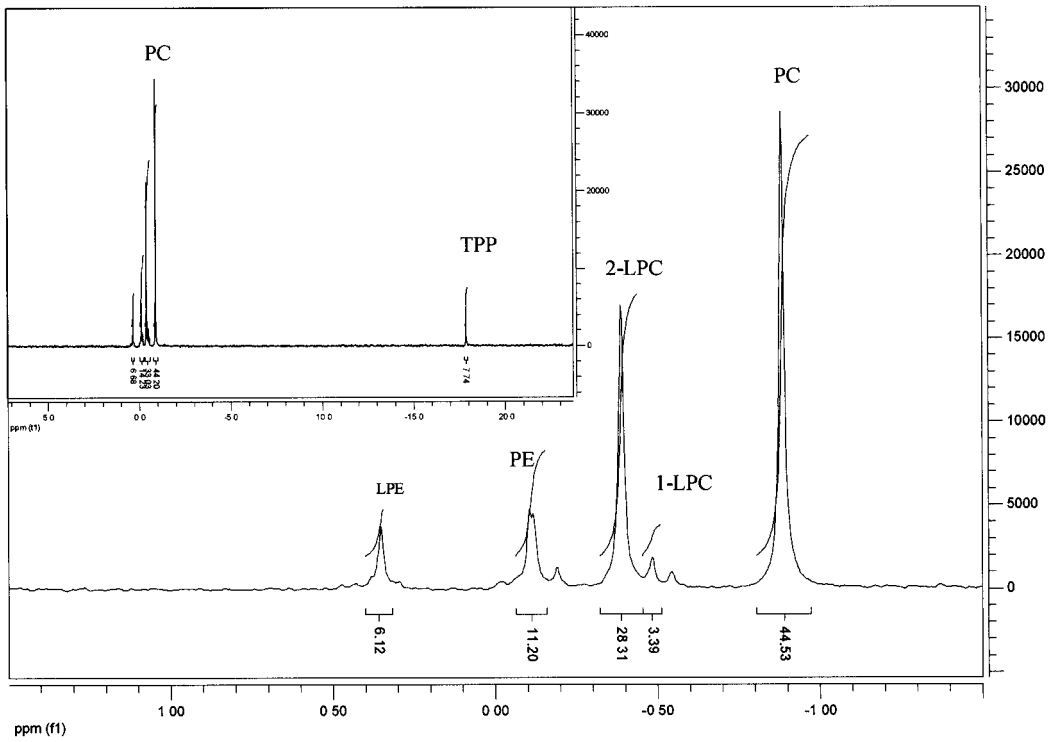


Figure 3: ^{31}P NMR spectrum of sample H2771-9 – LBJ#2344-85-4 30. The zoomed spectrum [-1.5 – 1.5ppm] shows the chemical shifts of the phospholipids as well as the integrals. The inserted spectrum is the full spectrum [-23.7 – 7.2ppm] with the internal standard TPP* at δ= -17.84ppm (corrected integral value 7.74).Nomenclature: 2-LPC: 1-acyl-sn-glycero-3-phosphorylcholine.

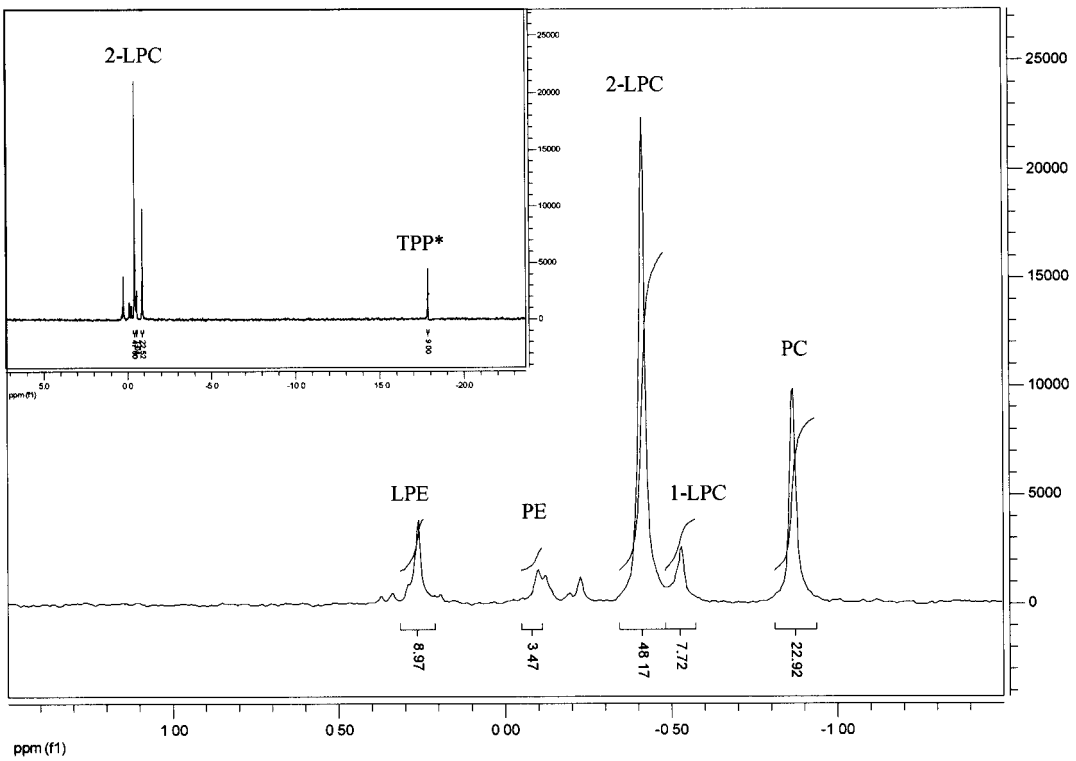


Figure 4: ^{31}P NMR spectrum of sample H2771-11 – LBJ#2344-85-4 120. The zoomed spectrum [-1.5 – 1.5ppm] shows the chemical shifts of the phospholipids as well as the integrals. The inserted spectrum is the full spectrum [-23.7 – 7.2ppm] with the internal standard TPP at δ= -17.84ppm (corr. integral value 9.00).

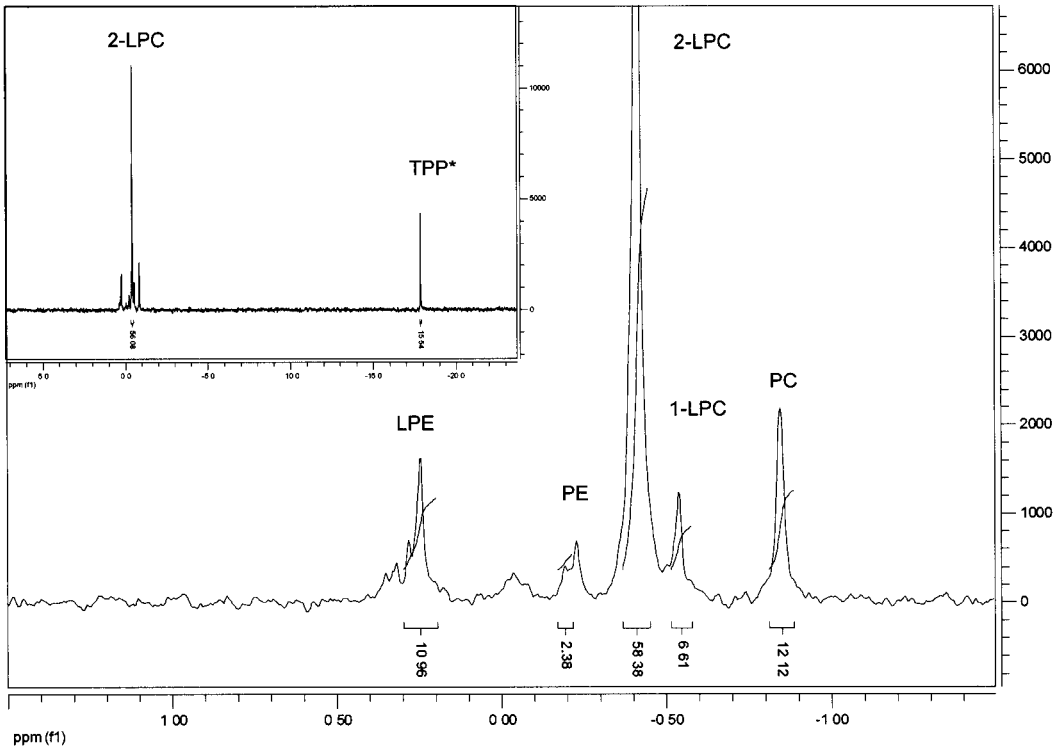


Figure 5: ^{31}P NMR spectrum of sample H2771-14 – LBJ#2344-85-4 360. The zoomed spectrum [-1.5 – 1.5ppm] shows the chemical shifts of the phospholipids as well as the integrals. The inserted spectrum is the full spectrum [-23.7 – 7.2ppm] with the internal standard TPP at δ= -17.84ppm (corr. integral value 15.54).

Table 7: Percentage molar distribution of phospholipid from enzymatic treatment of egg yolk with KLM3' dosed at 2.0 LATU/-g yolk at 50°C. Amount internal standard TPP was 3.9 μmol.

Sample ID	Incubation time	g yolk	TPP*	PC	1-LPC	2-LPC	PE	LPE
2344-85-4-0	0 min	1.030	7.93	73.71	0.0	2.44	17.49	1.27
2344-85-4-30	30 min	1.067	7.74	44.53	3.39	28.31	11.20	6.12
2344-85-4-120	120 min	1.052	9.0	22.92	7.72	48.17	3.47	8.97
2344-85-4-360	360 min	0.996	15.54	12.12	6.61	58.38	2.38	10.96

*The value listed for TPP is the corrected integral value.

The increase in molar percentage of the internal standard TPP over time (from 7.93 to 15.54 for series 2344-85-4) indicated loss of phosphorous from the system. In the egg yolk system studied, loss of phosphorous was most likely caused by the formation of glycerophospholipid (GPC). Besides being an acyltransferease, see Figure 2, KLM3' was demonstrated to also to have lyso-phospholipid activity thereby hydrolysing LPC to GPC, see Figure 6. Being water-soluble, GPC was not detectable in the organic phase analysed by ^{31}P NMR. Presenting conversion degree of phospholipid to lyso-phospholipd (Sum of percentage LPC+LPE at time t=x divided by the sum of percentage PC+PE at time t=0) based on relative molar distribution gave a wrong indication, since the conversion degree was seen to increase over time, see Figure 7. Because part of the lyso-phosholipid formed by the acyltranseferase reaction was degraded by the lyso-phospholipid reaction, the conversion degree based on absolute data (Sum of μmol LPC+LPE at time t=x

divided by sum of μmol PC+PE at time t=0) first increased and then decreased as incubation time elapsed, see Figure 7.

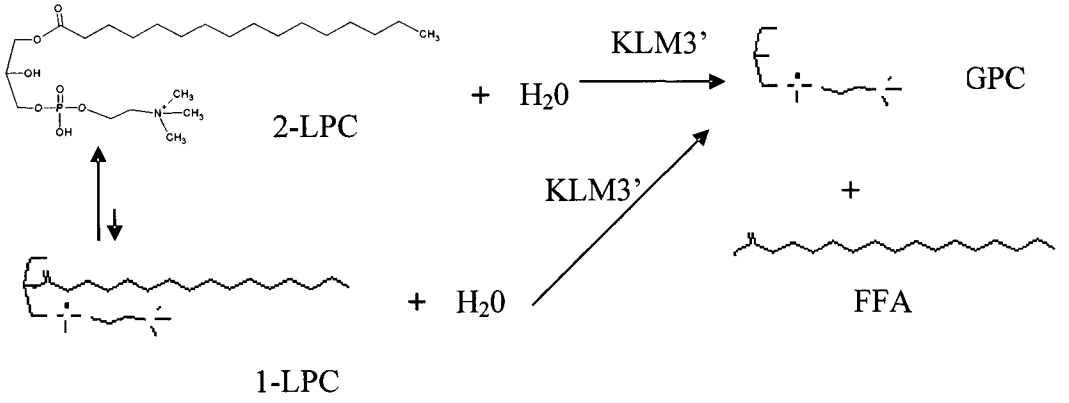


Figure 6: The acyltransferase KLM3' also has lyso-phospholipid activity thereby degrading LPC to GPC with contaminant formation of a free fatty acid.

Assuming the amount of internal standard (TPP) and the phospholipid that is removed during the CsEDTA wash was the same in all samples, the amount of μmol of each phospholipid type in the 800 μl $CDCl_3$/MeOH extract was calculated by relating the known amount of internal standard added to the molar percentage of the internal standard in a given sample, see Equation 1. No calculation back to the original sample was made due to unknown distribution between organic and water phases at numerous extractions during sample preparation. Assuming though that distribution between the two phases was the same in all samples, the amount μmol in 800 μl $CDCl_3$/MeOH extract was adjusted for the exact amount of sample withdrawn from the egg yolk, see Equation 1.

Results are expressed as percentage phospholipid conversion based on μmol 1-LPC+2-LPC+LPE at a given time relative to μmol PC+PE at time t=0 min present in 800μl $CDCl_3$/MeOH NMR sample adjusted for the exact amount sample withdrawn from the egg yolk, see Equation 2.

Equation 1: Calculation of μmol of a given component.

$$\mu mol\ X = \frac{\mu mol\ TPP \cdot}{TPP_{molar\ \%}} \cdot X_{molar\ \%}$$

Equation 2: Calculation of % phospholipid conversion based on absolute data. Percentage molar distribution data are taken from Table 7.

$$\%Phospholipid\ conversion = \frac{\mu mol\ TPP_{t=x} \cdot \dfrac{1 - LPC_{molar\%\ at\ t=x} + 2 - LPC_{molar\%\ at\ t=x} + LPE_{molar\%\ at\ t=x}}{TPP_{molar\%\ at\ t=x} \cdot g\ yolk_{t=x}}}{\mu mol\ TPP_{t=0} \cdot \dfrac{PC_{molar\%\ at\ t=0} + PE_{molar\%\ at\ t=0}}{TPP_{molar\%\ at\ t=0} \cdot g\ yolk_{t=0}}} \cdot 100\%$$

Internal standard TPP = 3.9 µmol

$$\%Phospholipid\ conversion\ 30\ \text{min} = \frac{3.9\ \mu mol \cdot \dfrac{3.39 + 28.31 + 6.12}{7.74 \cdot 1.067g}}{3.9\ \mu mol \cdot \dfrac{73.41 + 17.49}{7.93 \cdot 1.030g}} \cdot 100 = 41.1\%$$

$$\%Phospholipid\ conversion\ 120\ \text{min} = \frac{3.9\ \mu mol \cdot \dfrac{7.72 + 48.17 + 8.97}{9.00 \cdot 1.052g}}{3.9\ \mu mol \cdot \dfrac{73.41 + 17.49}{7.93 \cdot 1.030g}} \cdot 100 = 61.6\%$$

$$\%Phospholipid\ conversion\ 360\ \text{min} = \frac{3.9\ \mu mol \cdot \dfrac{6.61 + 58.38 + 10.96}{15.54 \cdot 0.996g}}{3.9\ \mu mol \cdot \dfrac{73.41 + 17.49}{7.93 \cdot 1.030g}} \cdot 100 = 44.0\%$$

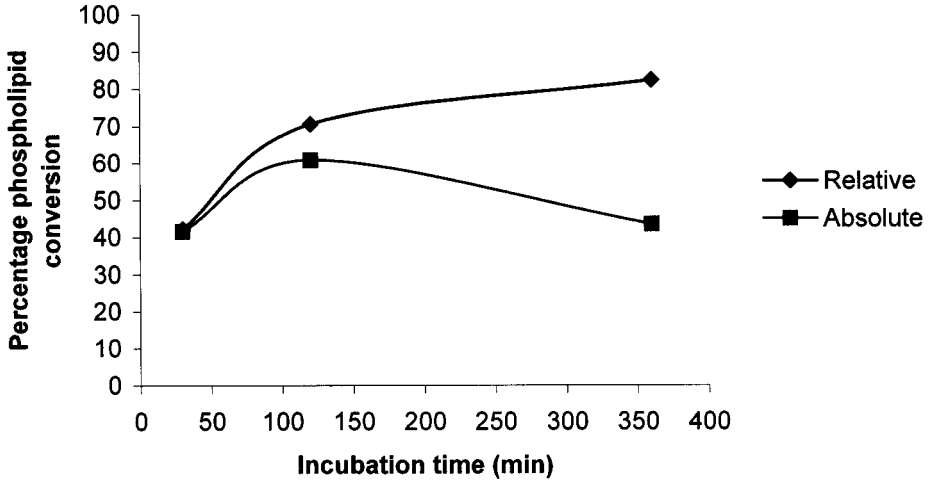


Figure 7: Percentage phospholipid conversion based on relative (percentage molar distribution) and absolute (µmol) data, respectively.

The first parameter evaluated was pH. Egg yolk has a natural pH of approximately 6.0 and the optimum pH for KLM3' was found to be between 7.0 and 10.0, peaking at pH 8.0. Therefore, an initial pH adjustment of the egg yolk to a more alkaline pH value could give a higher conversion of phospholipid to lysophospholipid applying the same enzyme dosage. Effect of pH on phospholipid conversion in egg yolk can be seen in Figure 8. The enzyme dosage used was 2.0 LATU/g yolk and incubation time was 2 hours at 50°C.

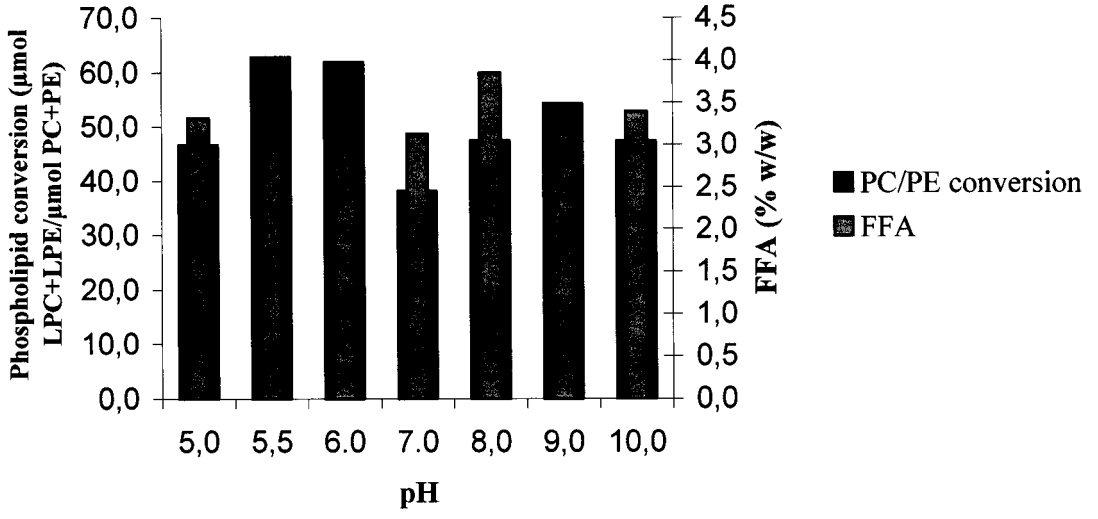


Figure 8: Effect of pH on phospholipid conversion in egg yolk with KLM3' dosed at 2.0 LATU/g yolk. Incubation temperature was 50°C and incubation time was 2 hours.

As seen in Figure 8, the optimum conversion of phospholipid is obtained at pH 6.0, the natural pH of egg yolk. At pH 6.0, the ratio between free fatty acid and the phospholipid conversion is the lowest. This is preferable, since free fatty acid contributes to the oxidation instability of the end product, mayonnaise. Based on these observations, natural egg pH, 6.0, was chosen as the pH value for the remaining part of the kinetic study. From an industrial point of view, running

the enzymatic treatment of egg yolk at its natural pH value is also preferable, as a pH adjustment would add another process step.

Next, the effect of temperature in combination with enzyme dosage as well as incubation time was evaluated. KLM3' has a temperature optimum of 65°C but as heat inactivation of the enzyme at temperatures of 60°C and above are pronounced and enzymatic treatments ran for up to six hours, enzymatic treatments were run at 50°C, 55°C and 60°C. The high temperature (65°C) at long holding times (six hours) could also very well affect the egg yolk.

For enzymatic treatments run at 50°C, the percentage phospholipid conversion as well as percentage FFA and cholesterol as a function of enzyme dosage are seen in Figure 9. Irrespective of the enzyme dosage used or the incubation time elapsed, the maximum percentage phospholipid conversion reached was approximately 60%. The percentage FFA increased steadily with increasing enzyme dosage and longer incubation time. Within the first 60 minutes incubation, 90% of the cholesterol was eliminated for all enzyme dosages. At incubation times longer than 60 minutes, no further change in cholesterol level was observed for any enzyme dosage. The 90% reduction in cholesterol level within the first 60 minutes of incubation showed the acyltransferase reaction of KLM3' to be fast and efficient as long as a certain level of cholesterol was still present.

For the lowest dosage, 1.5 LATU/g yolk tested at 50°C, a plateau close to 60% phospholipid conversion was observed at incubation times between 120 and 360 minutes. During the same period of time a steady increase in FFA was observed. This indicated a steady state at which the amount of LPC formed by hydrolysis of PC equalled the amount of LPC degraded by the lyso-phospholipid reaction. At the two highest enzyme dosages, 3.0 and 5.0 LATU/g yolk tested at 50°C, a significant decrease in percentage phospholipid conversion was observed at long incubation times. At these dosages and times, the lyso-phospholipid reaction exceeded the phospholipid hydrolysis reaction leading to a reduction in the amount of LPC present.

In Figure 10, the percentage phospholipid conversion as well as percentage FFA and cholesterol as a function of enzyme dosage for enzymatic treatments run at 55°C can be seen. As was the case for enzymatic treatments run at 50°C, the maximum percentage conversion of phospholipid obtained at 55°C was 60% regardless of the enzyme dosage used or the incubation time elapsed. Percentage FFA increased with increasing enzyme dosage and longer incubation times. At 55°C, 90% of the cholesterol was eliminated within the first 30 minutes of incubation as opposed to 60 minutes at 50°C; hence the acyltransferase reaction of KLM3' is even faster at 55°C than at 50°C.

For enzymatic treatments run at 55°C, the three lowest dosages tested, 0.5, 1.0, and 1.5 LATU/g yolk, a plateau close to 60% phospholipid conversion was observed for incubation times between 60 and 360 minutes. During the same time interval, a steady increase in FFA was observed. In Figure 12, the amount of μmol PC and LPC is seen for the enzymatic treatment with KLM3' dosed at 0.5 LATU/g yolk. PC was steadily degraded for the first 240 minutes incubation time, whereas the accumulation of LPC ceased after 60 minutes incubation time, showing that the LPC formed by hydrolysis of PC equalled the amount of LPC degraded by the lyso-phospholipid reaction for incubation times between 60 and 240 minutes. During this period of time no change in the total amount of LPC was observed, but as the total amount of PC decreased with time, so did the total emulsification capacity of the egg yolk, because PC as opposed to GPC is an emulsifier.

At the higher dosages tested at 55°C, reduction in phospholipid conversion was observed at prolonged incubation times, indicating the lyso-phospholipid reaction to exceed the phospholipid reaction. This reduction was not as pronounced at 55°C as at 50°C, presumably because the enzyme was partly inactivated at the higher temperature at long holding times.

In Figure 11, the percentage phospholipid conversion as well as percentage FFA and cholesterol as a function of enzyme dosage for enzymatic treatments run at 60°C is seen. As for enzymatic treatments run at 50°C and 55°C, the maximum percentage conversion of phospholipid obtained at 60°C was 60% irrespective of the enzyme dosage used or the incubation time elapsed. For all dosages tested at 60°C, the phospholipid conversion peaked within the first 60 minutes of incubation time. At incubation times longer than 120 minutes, no further change was observed in percentage phospholipid conversion within each dosage tested. Percentage FFA increased for the first 120 minutes incubation time and then ceased. This combined with the plateau of percentage phospholipid conversion for each dosage at incubation times above 120 minutes indicated pronounced heat inactivation of the enzyme. At 60°C, 90% of the cholesterol was eliminated within the first 30 minutes of incubation as was the case for enzymatic treatments at 55°C.

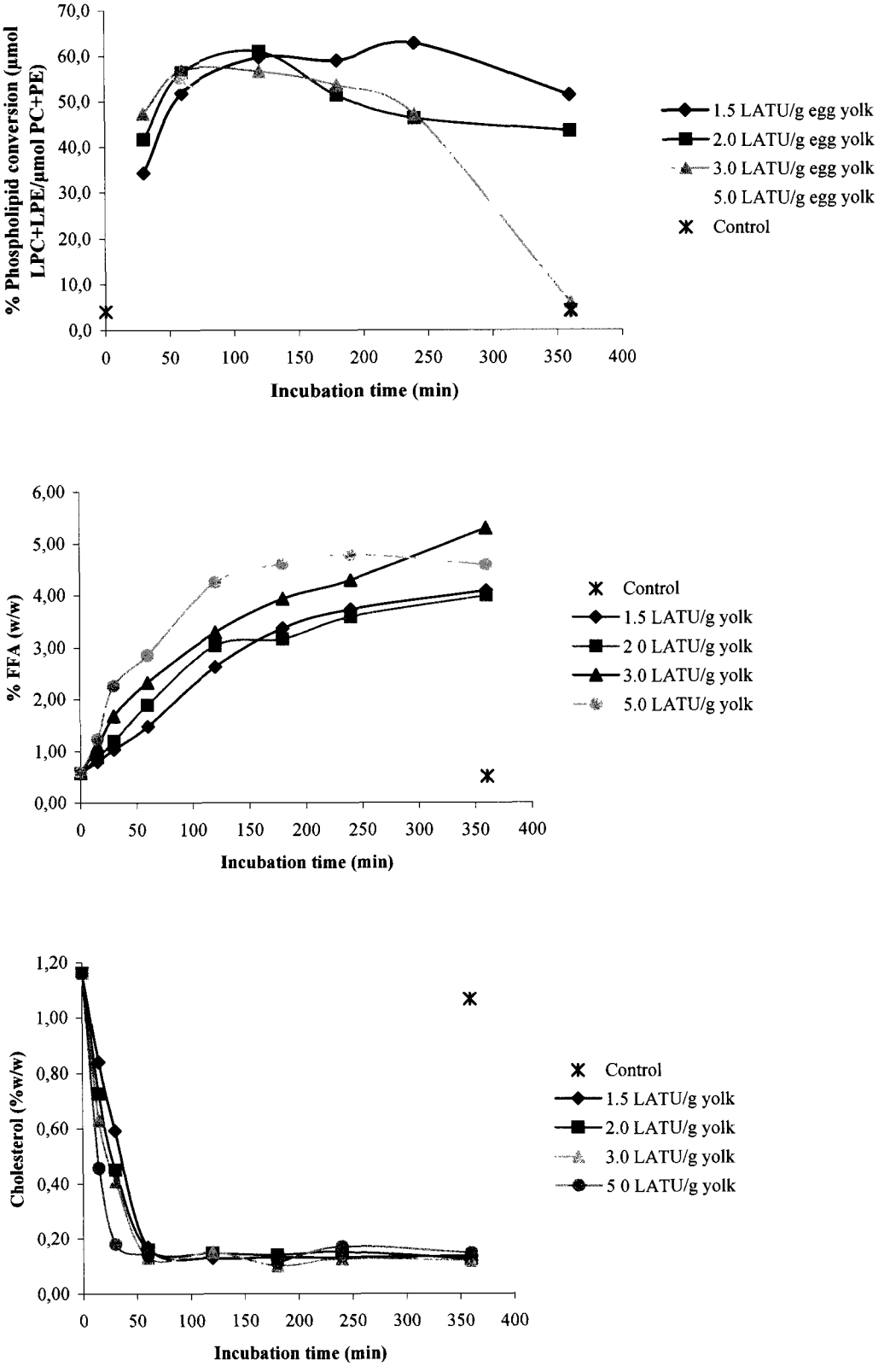
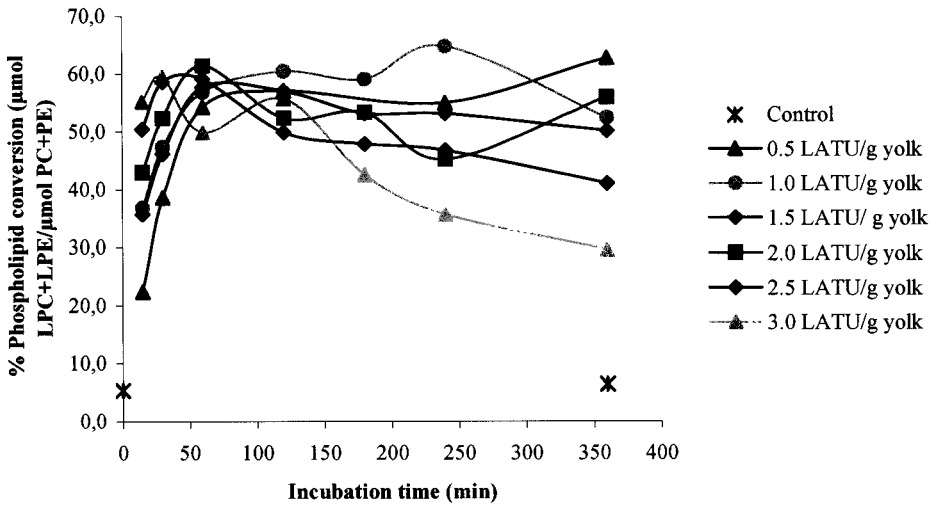
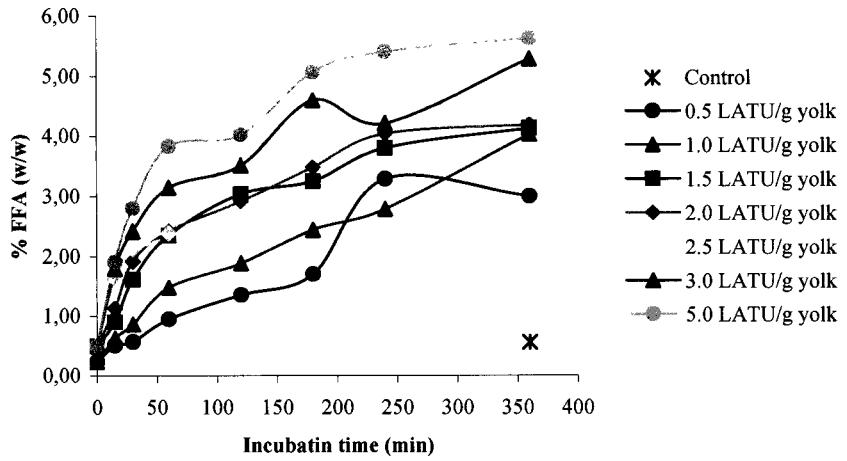


Figure 9: Enzymatic modification of egg yolk with KLM3' acyltransferase at 50°C and natural egg pH. Phospholipid conversion is expressed at µmol LPC+LPE at a given time relative to µmol PC+PE at time t=0 present in 800 µl CDCl3/MeOH NMR sample. Free fatty acid and cholesterol are expressed as percentage of egg yolk.





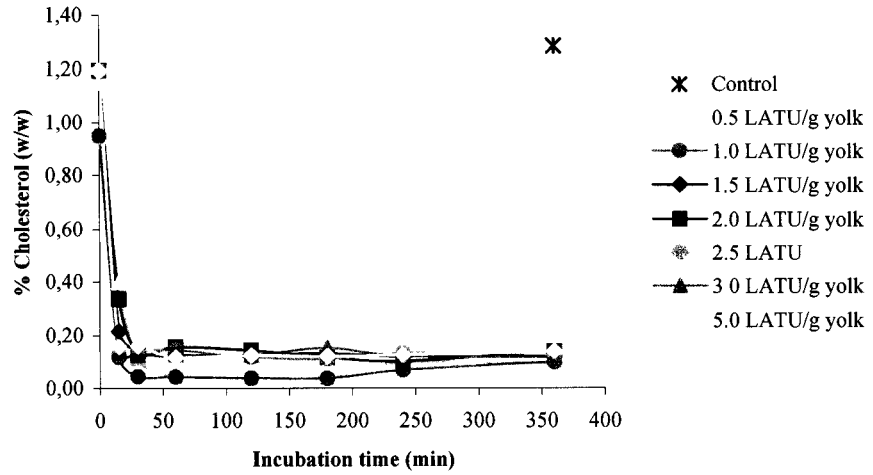


Figure 10: Enzymatic modification of egg yolk with KLM3' acyltransferase at 55°C and natural egg pH. Phospholipid conversion is expressed at µmol LPC+LPE at a given time relative to µmol PC+PE at time t=0 present in 800 µl CDCl3/MeOH NMR sample. Free fatty acid and cholesterol are expressed as percentage of egg yolk.

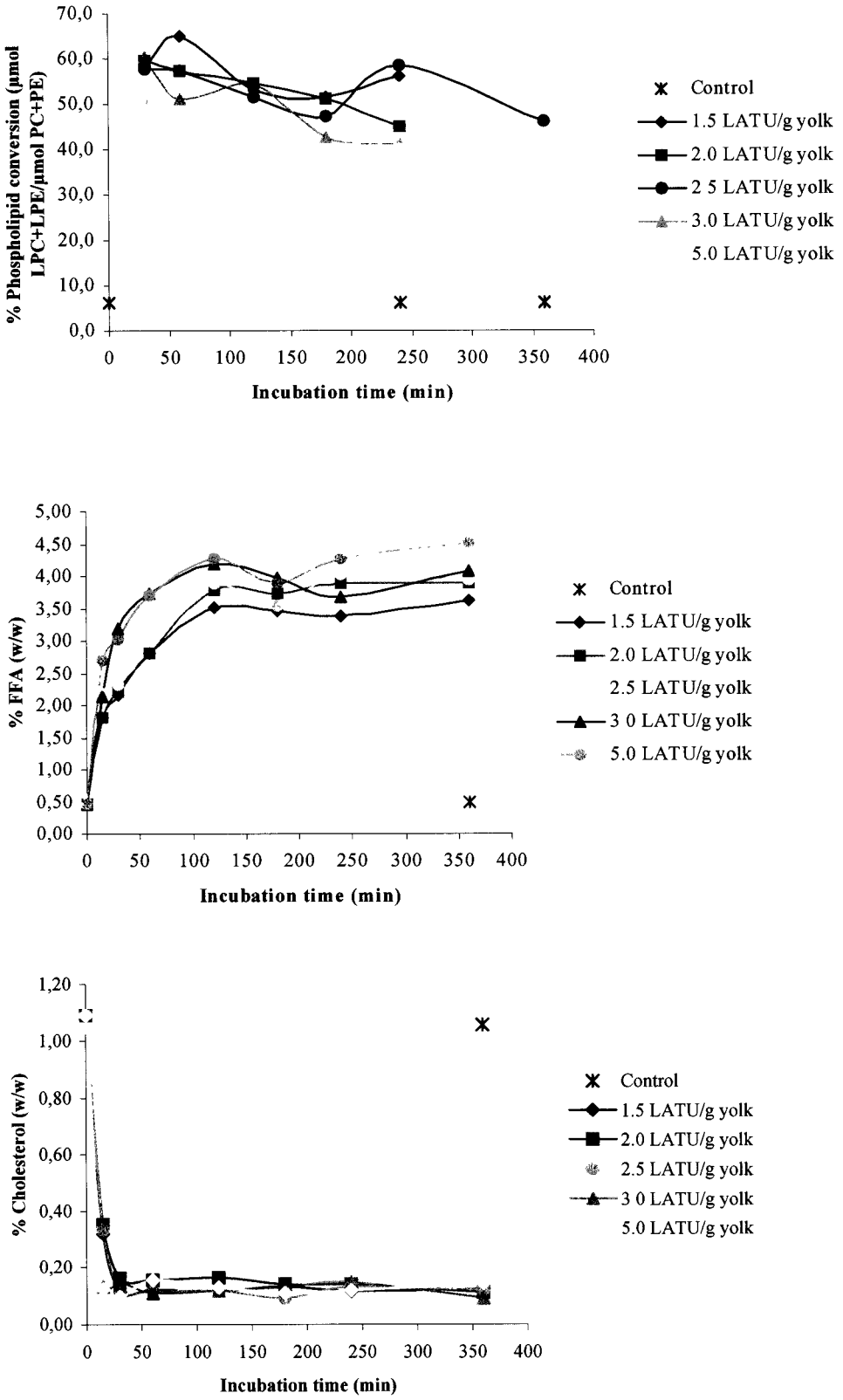


Figure 11: Enzymatic modification of egg yolk with KLM3' acyltransferase at 60°C and natural egg pH. Phospholipid conversion is expressed at µmol LPC+LPE at a given time relative to µmol PC+PE at time t=0 present in 800 µl CDCl3/MeOH NMR sample. Free fatty acid and cholesterol are expressed as percentage of egg yolk.

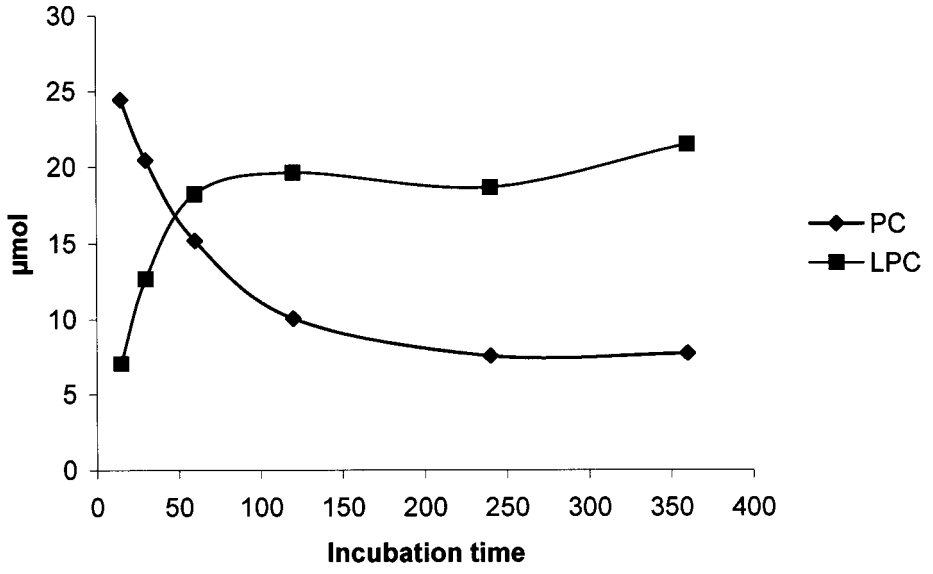


Figure 12: µmol PC and LPC as a function of incubation time for enzymatic treatment at 55°C with KLM3' dosed at 0.5 LATU/g yolk. PC was degraded for incubation times until 240 minutes, whereas the accumulation of LPC ceased after 60 minutes incubation time, showing the lyso-phopholipid reaction to be equal to the phospholipid reaction at incubation times between 60 and 240 minutes.

Enzymatic conversion of lecithin to lyso-lecithin improves the emulsification properties of egg yolk. These improved emulsification properties of egg yolk are of importance when making a.o. mayonnaise by preventing separation of the product upon pasteurisation. To evaluate the effect of enzymatic modification of egg yolk, pilot scale mayonnaises were made. Four sets of pilot scale mayonnaises were made using egg yolks treated with varying enzymes, dosages, and incubation times, and temperatures, see Table 2 and Table 5. Full fat mayonnaise made with unmodified egg yolk has an egg yolk content of 5.0%. Within each of the four sets of mayonnaises, 2% of egg yolk was tested to evaluate to which level the egg content in mayonnaise can be reduced by using enzymatically modified egg yolk. By measuring viscosity and particle size distribution of the pilot scale mayonnaises, the emulsification property of the enzyme treated egg yolk used was monitored, see Modification of egg yolk with KLM3'

. Particle size distribution data was also used for evaluation of the heat stability of the mayonnaises as well as visual judgement of oiling- out upon heat treatment. As opposed to applying conventional phospholipases for modification of egg yolk, KLM3', will give a lower level of free fatty acid due to the formation of cholesterol ester. Therefore the correlation between amount FFA present in enzyme treated egg yolk and oxidation stability of the pilot scale made mayonnaises was also evaluated, see Modification of egg yolk with KLM3'

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Mayonnaise ID	Enzyme used	% egg yolk	% PC conversion	% FFA	Particle size (% particles with diameter below 10 µm)			Viscosity (cp)	Oxidation stability (h)
					No treatment	Shaken	Heated		
FM06-31-2	None	2.5	5	0.4	51.6	53.8	nd	225000	11.9
FM06-31-4	KLM3'	2.5	54	4.1	58.6	43.6	nd	788000	8.4
FM06-31-6	Lysomax PLA2	2.5	75	2.5	85.0	63.6	nd	706000	8.9
FM06-31-8	Lipomod 699L	2.5	94	2.0	85.5	82.1	nd	850000	7.4
FM06-31-10	HSEYP	2.5	77	nd	64.5	56.0	nd	758000	7.8
FM06-31-14	KLM3	1.5	25	2.5	43.5	32.2	nd	347000	9.4
FM06-31-16	KLM3	1.5	19	4.2	45.3	29.4	nd	233000	8.2
FM06-31-18	Lipomod 699L	1.5	94	2.2	54.2	42.1	nd	282000	8.8
FM06-31-20	None	1.5	5	0.5	27.0	23.3	nd	66000	nd
FM06-31-22	HSEYP	1.5	77	nd	nd	nd	nd	280000	nd
FM06-31-31	HSEYP	1.5	77	nd	48.4	31.1	nd	259000	8.3
FM06-31-32	HSEYP	2.0	77	nd	nd	nd	nd	255000	nd
FM06-31-32	HSEYP	2.5	77	nd	nd	nd	nd	273000	nd
FM06-31-40	KLM3'	1.5	57	1.6	64.5	61.9	54.0	499000	8.6
FM06-31-43	Lipomod 699L	1.5	>95	3.0	74.3	76.1	73.0	555000	9.0
FM06-31-45	Lipomod 699L+none	2.0+1.0	60*	2.2	95.1	93.8	95.6	864000	7.5
FM06-31-46	HSEYP	1.5	77	nd	53.4	59.5	58.1	519000	8.9

Table 8: Pilot scale made mayonnaises
*Estimated based on 67% Lipomod 699L treated egg yolk with >95% PC conversion plus 33% raw egg yolk.

Besides KLM3', two other enzymes, Lipomod 699L (commercial phospholipase A_2 from Biocatalyst sold to the egg industry) and Lysomax PLA2 (commercial phospholipase A_2 from Genencor sold to the egg industry) were tested in the first set of mayonnaises made. As reference/market standard, HSEYP was used, a commercial enzymatically modified egg yolk product from Sanovo A/S. The first set of mayonnaises was made with 2.5% and 5.0% egg yolk. For the mayonnaises made with 5% enzyme treated egg yolk, the viscosity was above the upper detection limit and hence the mayonnaises were too viscous. In comparison, the mayonnaise made with 5% untreated egg yolk had a viscosity close to one third of the viscosity of the mayonnaises made with 5% enzymatically treated egg yolk (369000 cp vs.>999999 cp). Mayonnaise made with 2.5% unmodified egg yolk had a viscosity of 225000 cp. Mayonnaises made with 2.5% egg yolk modified with either KLM3', Lipomod 699L or Lysomax PLA2 corresponding to a PC conversion of 54%, 94% and 75%, respectively, had viscosities ranging from 700000 to 850000cp, see Figure 13. Based on mayonnaises made with 2.5% enzymatically modified egg yolk, no significant change in viscosity was observed for PC conversion ratios between 50 and 100%. Looking at particle size distribution for mayonnaises made with 2.5% egg yolk, no clear trend between this and the PC conversion ratio of the egg yolk seems obvious, see Figure 14. The change towards a lower percentage of particles with a diameter below 10 μm upon shaking the mayonnaises was most pronounced for PC conversions between 50% and 80%, but within this range no correlation between the drop in percentages particles with diameter below 10 μm and the degree of PC conversion was seen. For the high PC conversion of 94%, only a negligible drop in percentages particle with diameter below 10 μm was seen. Upon heat treatment, weak oiling-out was observed for mayonnaise made with unmodified egg yolk whereas no oiling-out was observed for any of the mayonnaises made with enzymatically modified egg yolk. Thus, mayonnaise with 2.5% KLM3' modified egg yolk is heat stable and viscosity-wise on level with market standard 2.5% HSEYP mayonnaise as well as with Lipomod 699L and Lysomax PLA2 mayonnaise, and particle size-wise on level with market standard HSEYP mayonnaise as well as with Lysomax PLA2 mayonnaise.

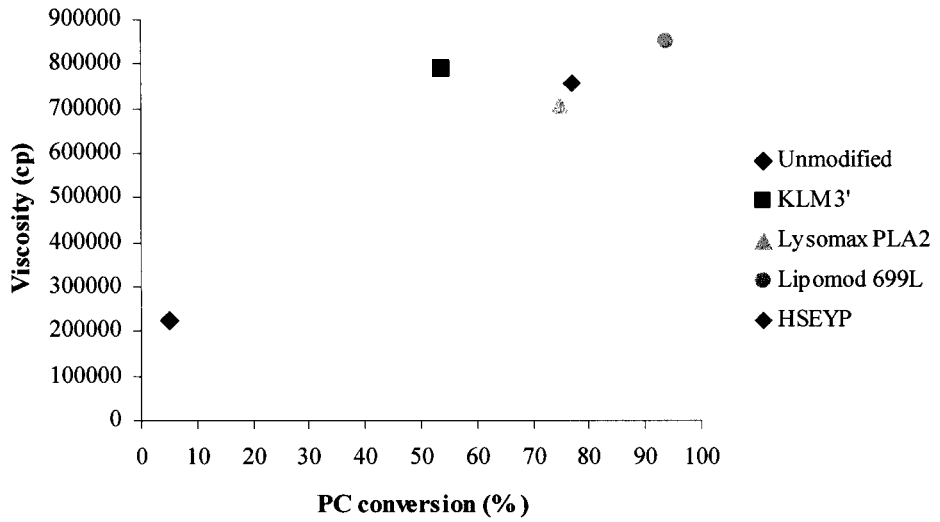

Figure 13: Viscosity of pilot scale mayonnaises as a function of PC conversion of the enzyme modified egg yolk. Mayonnaises were made with 2.5% egg yolk (data from set 1).

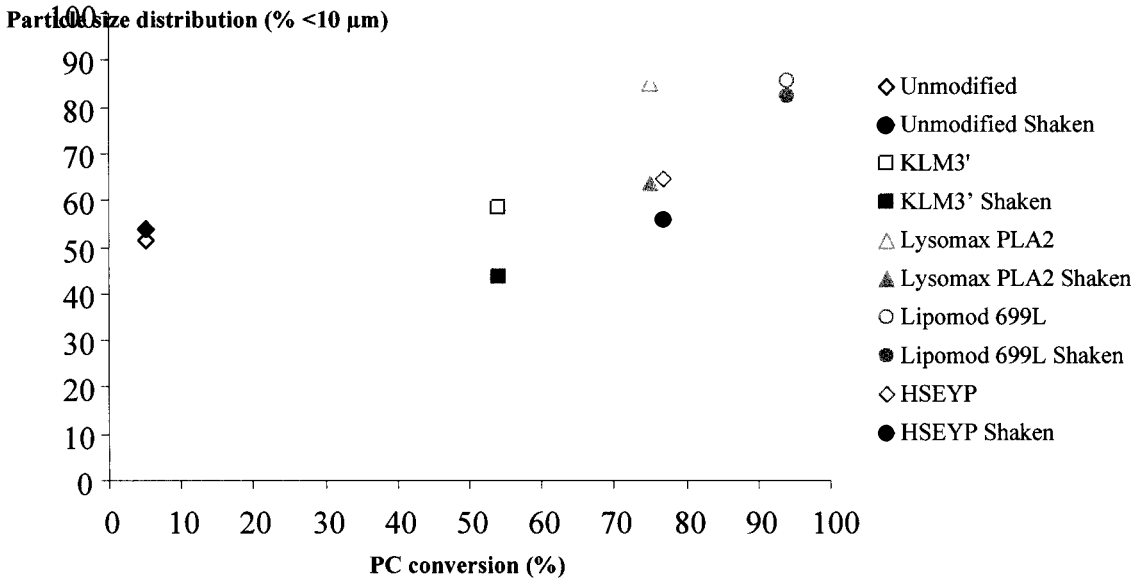

Figure 14: Particle size distribution of pilot scale mayonnaises as a function of PC conversion of the enzyme modified egg yolk. Mayonnaises were made with 2.5% egg yolk. Mayonnaise kept at 4-8°C for 72 hours as well as mayonnaise shaken at 300 rpm at room temperature for 72 hours was measured (Data from set 1).

The second set of mayonnaises was made without stabilizer and with 1.5% and 3.0% egg yolk. The enzyme modification of the egg yolks used in the second set of mayonnaises was carried out in a water bath as one batch for each enzyme, whereas the egg yolks used for the other three sets of mayonnaises were modified in a heating block as several batches for each enzyme and then pooled before use. The modification of egg yolk with KLM3' (1.5 LATU/g yolk) for both 60 minutes and 240 minutes caused LPC degradation due to the LPC activity of KLM3' and hence

the PC conversion was reduced to 25% and 19%, respectively. The free fatty acid content of these egg yolks also reflected the LPC degradation as the egg yolk modified with KLM3' for 60 minutes contained 2.5% FFA and the 240 minutes contained 4.2% FFA even though the latter had the lowest PC conversion of the two. Difference in the FFA content of egg yolk modified with KLM3' for 60 or 240 minutes was not reflected in the oxidation stability of mayonnaises made with 3% egg yolk, as the oxidation stability of the two mayonnaises was the same despite the fact that one contained almost twice as much FFA as the other, see Figure 15. Possibly, the difference in FFA content was masked by antioxidant in the oil used for mayonnaise. Due to the lack of stabilizers, the viscosity of the mayonnaises with 3.0% egg yolk was halved compared to the mayonnaises made with 2.5% egg in the first set of mayonnaises (approximately 400000 cp versus approximately 800000 cp). As for the first set of mayonnaises, no significant change in viscosity was observed for a broad PC conversion interval (25% to 94% PC conversion), see

The third set of mayonnaises was also made without stabilizer and with 1.5%, 2.0%, 2.5%, and 3.0% HSEYP egg yolk in order to set the egg content for future pilot scale mayonnaise productions. In all cases, the viscosity of the four mayonnaises was within a range of 255000 to 302000cp. Mayonnaise with an egg content of 1.5% and 3.0% was chosen for further study.

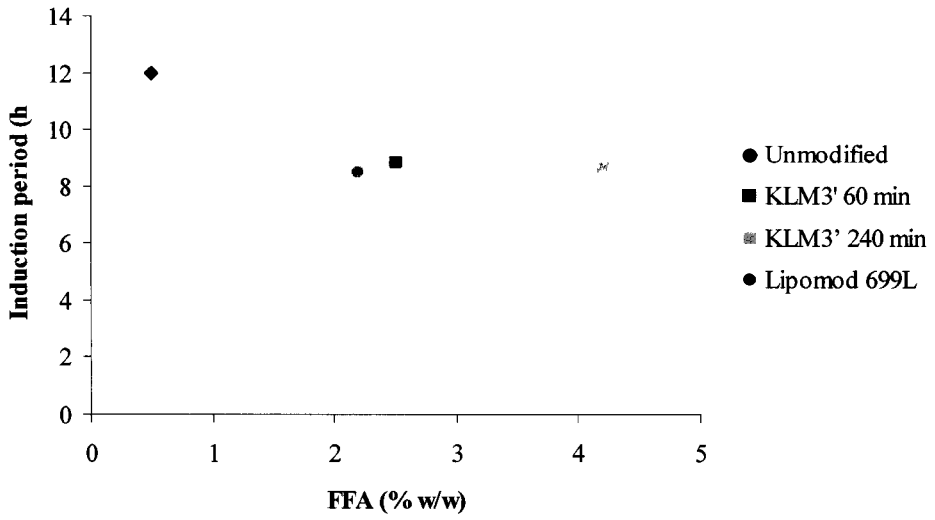

Figure 15: Oxidation stability of mayonnaise as a function of percentage FFA in egg yolk. Mayonnaises were made with 3.0% egg yolk and no stabilizer (Data from set 2).

In the fourth set of mayonnaises, egg yolks modified with KLM3' or Lipomod 699L were used as well as HSEYP. Also, two part Lipomod 699L modified egg yolk blended with one part unmodified egg yolk was used for pilot scale mayonnaise production. The viscosity of the four mayonnaises made with 3.0% egg yolk was in the range 790000 cp to 934000 cp, see Figure 16, which was on level with the mayonnaises made with 2.5% egg yolk in the first set (700000cp to 850000 cp). As was the case for mayonnaises made with 2.5% enzymatically modified egg yolk, no significant change in viscosity was observed for PC conversion ratios between 50 and 100% for the mayonnaises made with 3.0% modified egg yolk. As expected, the viscosity of mayonnaises made with 1.5% egg yolk was lower than the viscosity of mayonnaises made with 3.0% egg yolk, 499000 cp to 555000 cp versus 790000 cP to 934000 cP, see Figure 17. For

mayonnaises made with 1.5% egg yolk, a gentle increase in viscosity was seen with the PC conversion ratio ascending from 57% to >95%, see Figure 17. Still the overall increase in viscosity does not exceed 10%.

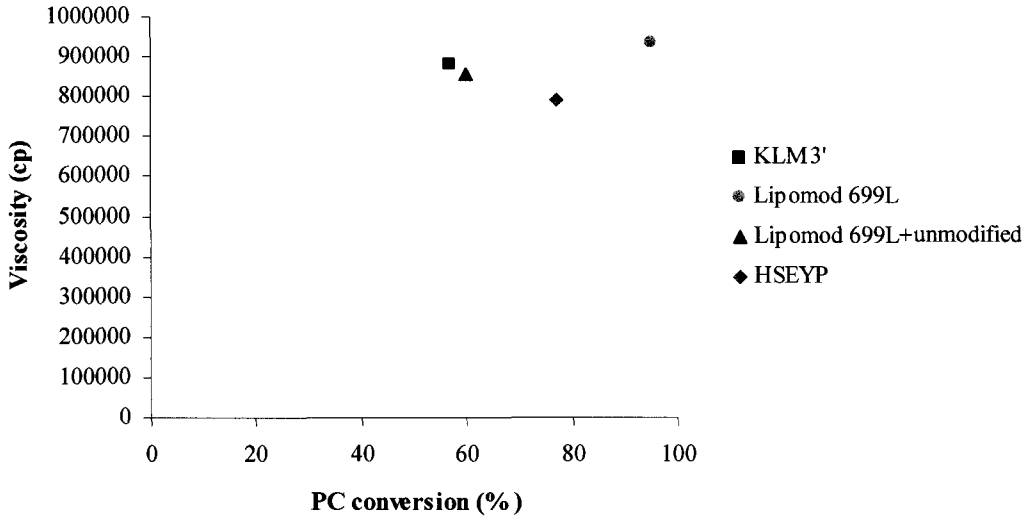

Figure 16: Viscosity of pilot scale mayonnaises as a function of PC conversion of the enzymaticaly modified egg yolk. Mayonnaises were made with 3.0% egg yolk (Data from set 4).

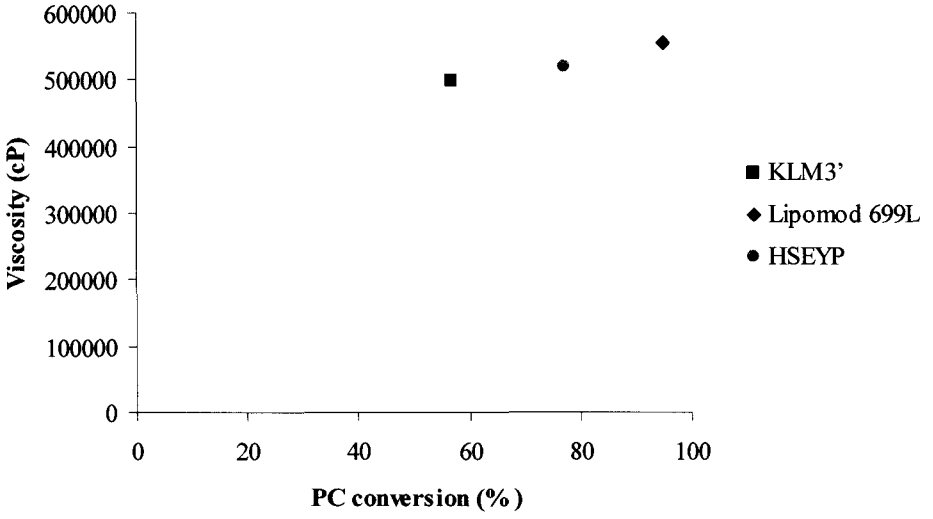

Figure 17: Viscosity of pilot scale mayonnaises as a function of PC conversion of the enzyme modified egg yolk. Mayonnaises were made with 1.5% egg yolk (Data from set 4).

The heat stability of the mayonnaises in the fourth set was tested by measuring particle size distribution before and after heat treatment (1 hour at 90°C), see Figure 18. Before heat treatment all mayonnaises made with 3.0% egg yolk had more than 93% particles with a diameter below 10 μm, indicating a fine/delicate emulsion also reflected by the high viscosities of these mayonnaises. Upon heating, only minor drops in percentage particles with a diameter below 10 μm was observed; hence the mayonnaises made with modified egg yolk were heat stable. Mayonnaise made with KLM3' treated egg yolk had the most pronounced drop in percentage

particles with a diameter below 10 μm (94% to 89.3%). Disregarding data from the mayonnaise made with Lipomod 699L treated egg yolk blended with unmodified egg yolk, it seemed that the lower the PC conversion ratio, the larger the drop in percentage particles with a diameter below 10 μm upon heating. The observations made for mayonnaises made with 3% egg yolk became more evident for mayonnaises made with 1.5% egg yolk. The lower viscosities of these 1.5% egg yolk mayonnaises and hence more coarse emulsions were reflected in the lower percentage particles with a diameter below 10 μm (less than 75% particles with a diameter below 10 μm for mayonnaise made with 1.5% egg yolk as opposed to at least 93% particles with a diameter below 10 μm for mayonnaises made with 3.0% egg yolk), see Figure 19. Mayonnaise made with 1.5% KLM3' modified egg yolk showed a significant drop in percentage particles with a diameter below 10 μm upon heating, whereas the mayonnaise made with Lipomod 699L modified egg yolk only showed a minor drop, see Figure 19. Hence for mayonnaise with low egg yolk content, a PC conversion ratio of 50% seemed on the low edge regarding heat stability. Still no oiling-out was observed upon heat treatment for any of the mayonnaises made with enzymatically modified egg yolk.

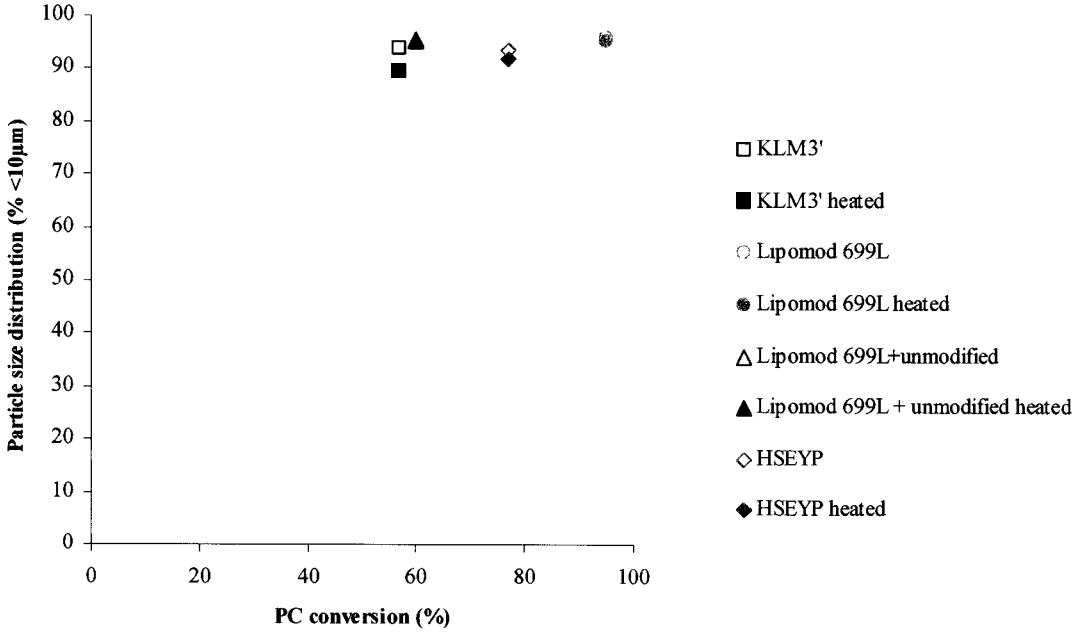

Figure 18: Particle size distribution of pilot scale mayonnaises as a function of PC conversion of the enzyme modified egg yolk. Mayonnaises were made with 3.0% egg yolk (Data from set 4). Mayonnaise kept at 4-8°C for 72 hours as well as mayonnaise kept at 90°C for one hour was measured.

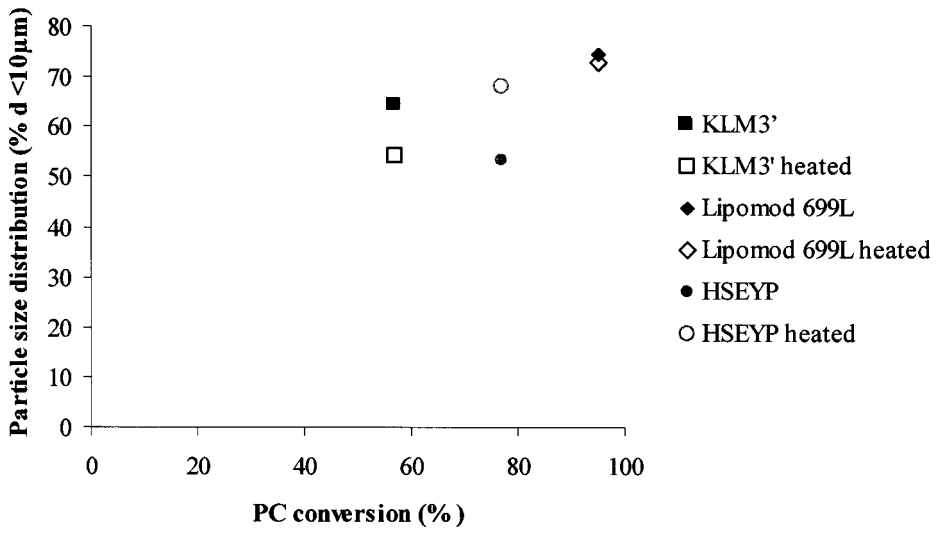

Figure 19: Particle size distribution of pilot scale mayonnaises as a function of PC conversion of the enzymatically modified egg yolk. Mayonnaises were made with 1.5% egg yolk (Data from set 4). Mayonnaise kept at 4-8°C for 72 hours as well as mayonnaise kept at 90°C for one hour was measured.

The amount of FFA in egg yolk modified with KLM3' was close to half the amount present in egg yolk modified with Lipomod 699L (corresponding to 57% PC conversion and >95% PC conversion respectively). However, this difference was not reflected in the oxidation stability of the mayonnaises, see Figure 20, as the induction period was approximately 8 hours for FFA contents between 1.6 and 3.0%. Again, as described for the second set of mayonnaises, the FFA content differences were most likely masked by antioxidant in the oil used for mayonnaise production. Based on the evaluation of the fourth set of mayonnaises, KLM3' modified egg yolk used to produce mayonnaise with an egg content of 3.0% gave viscosity and particle size distribution prior to heat treatment on level with the market standard HSEYP and Lipomod 699L modified egg yolk. With respect to heat stability, mayonnaise with an egg content of 3.0% made with Lipomod 699L modified egg yolk showed slightly better results than mayonnaise made with KLM3' modified egg yolk or HSEYP. This was more pronounced for mayonnaises made with an egg content of 1.5%.

The pilot scale mayonnaise trials have shown that the egg content in mayonnaise can be reduced from 5% as in a full fat mayonnaise to 2.5-3.0% by using enzyme modified egg yolk. Mayonnaise made with 2.5-3.0% enzymatically modified egg yolk had a higher viscosity as well as a larger amount of particles with a diameter below 10 µm both indicating a more delicate emulsion than mayonnaise made with unmodified egg yolk. Also the mayonnaises made with enzymatically modified egg yolk were heat stable as opposed to mayonnaise made with unmodified egg yolk that showed oiling-out upon heat treatment. The mayonnaises made with egg yolk modified with KLM3' were on level with mayonnaises made with egg yolk modified with Lysomax PLA2 or Lipomod 699L and HSEYP with respect to viscosity, particle size distribution, oxidation stability, and heat stability. However, mayonnaises made with an egg content of 1.5%, Lipomod 699L showed slightly better results than KLM3' regarding heat stability.

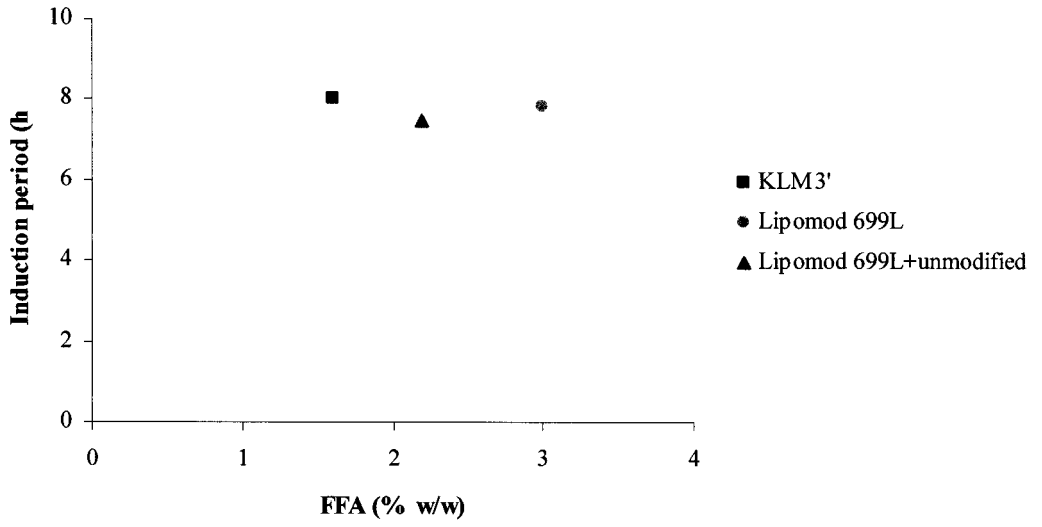

Figure 20: Oxidation stability of mayonnaise as a function of percentage FFA in egg yolk. Mayonnaises were made with 3.0% egg yolk (Data from set 4).

At the onset of the egg modifying project, the target was to develop an acyltransferase that was able to modify egg yolk on level with or better than Lecitase Ultra (competitor's product). The conversion of lecithin to lyso-lecithin makes it possible to produce heat stable mayonnaise. The ability of the acyltransferase to transfer the fatty acid moiety to cholesterol should give rise to a product less prone to oxidation, because of a reduced level of FFA. Based on a few application trials comparing mayonnaise made with KLM3' wild type and Lecitase Ultra, the KLM3' wild type met the above target.

Running the kinetic study to define the optimal conditions for PC conversion, it became evident that the maximum PC conversion degree obtainable with KLM3' regardless of the dosage, temperature and time was 60%. This level of PC conversion was demonstrated to be caused by KLM3' having LPC activity, which lead to degradation of lysolecithin.

CONCLUSION

A kinetic study with KLM3' in egg yolk was conducted to set the optimal parameters for PC conversion. Seven pH values in the range pH 5.0-10.0, three temperatures 50, 55, and 60°C, and seven enzyme dosages in the range 0.5 to 5.0 LATU/ g egg yolk were tested. Kinetics was followed by analysis of free fatty acid and cholesterol as well as by ^{31}P-NMR to monitor the PC/LPC level during modification. In all instances, cholesterol was eliminated within the first 30 minutes incubation time. Irrespective of the enzyme dosage, incubation temperature or time used, the maximum obtained PC conversion degree with KLM3' was 60%. KLM3' was demonstrated to have LPC activity causing degradation of already accumulated LPC and hence a low PC conversion ratio. The LPC activity of KLM3' was also reflected by elevated FFA amounts at low PC conversion degrees.

Pilot scale mayonnaise trials showed that KLM3' modified egg yolk was on level with mayonnaise made with Lysomax PLA2 or Lipomod 699L modified egg yolk, and with

mayonnaise made with HSEYP with regard to viscosity, particle size distribution, heat stability, and oxidation stability. Hence KLM3' modified egg yolk did not give a mayonnaise less prone to oxidation than mayonnaises made with conventional phospholipase modified egg yolk.
By using enzymatically modified egg yolk instead of unmodified egg yolk, the egg content in mayonnaise can be reduced by 50% from 5.0% to 2.5%.

3-20-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Vincent Sewalt [vincent.sewalt@danisco.com]
Sent: Friday, March 20, 2009 4:14 PM
To: West-Barnette, Shayla
Cc: Alice Caddow
Subject: Re: GRN 265
Attachments: GRN 265 clarification letter 032009.PDF

Dr. West-Barnette,

I have added the clarification you requested to Ms Caddow's original response letter, which I have attached here as a pdf file. Do I send the original by courier to you or to Dr. Martin ? I assume just the letter will be sufficient, or do we need to resubmit other portions of the dossier?

Best regards,

Vincent Sewalt, PhD.
Director, Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Office (650) 846-5861
Mobile (650) 799-0871

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"West-Barnette, Shayla"
<Shayla.WestBarnette@fda.hhs.gov>

To <vincent.sewalt@danisco.com>
cc
Subject GRN 265

Posted date : 03/19/2009 10:31:04 AM AST

Dr. Sewalt,

Thank you for taking the time to speak with me a few minutes ago. I have conferred with the members of the review team for GRN 265, and they had no additional comments other than what we spoke about

(regarding clarifying the different names used to refer to GCAT in the submission). If you have any questions, please feel free to contact me using the information below. Thanks,

Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (office)
Shayla.WestBarnette@fda.hhs.gov

GRN 265 Clarification Letter 032009 (3-20-09 rcd)

GENENCO
A Danisco Division

March 20, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Danisco US Inc.
925 Page Mill Road
Palo Alto, CA 94304
USA
Tel +1 650 846 7500
Fax +1 650 845 6505
www.genencor.com

RE: GRN 000265

Dear Dr. Martin:

We are writing in response to questions posed by FSIS and OFAS on the above GRAS Notice which were conveyed by phone conference with several OFAS staff, and email from Dr. Shayla West-Barnette, Consumer Safety Officer on March 12, 2009, and subsequent phone call and email form Dr. West-Barnett to Dr. Vincent Sewalt on March 19, 2009.

In response to Dr. West-Barnett's request to Dr. Sewalt, we wish to clarify that in several sections of the submission the GCAT (Glycerophospholipid Cholesterol Acyltransferase) enzyme is also referred to as KLM3', our pre-launch internal code name for this enzyme. We apologize for the inconsistency in referring to the same enzyme by two different names in this submission, but trust that this clarification suffices.

In response to the earlier request to clarify how the enzyme will be used to treat eggs, we offer the following. The action of the enzyme will be on egg yolk. For use of the egg yolk in applications such as mayonnaise, the eggs will we treated at an egg processing plant. After being separated from the egg whites, the yolks will be treated with the enzyme and pasteurized at 80° C or greater which inactivates the enzyme. They can then be sold as treated liquid yolk that is refrigerated until use or are spray dried into powder. For the baking application, the eggs will be treated by the bakery as whole eggs, but the intended effect is only on the yolk.

We have also included two studies conducted by Genencor, one on the treatment of liver sausage with KLM3' and the other on the treatment of egg yolk. Both studies demonstrate that the enzyme is effective at emulsifying the food substrate. The enzyme treated lever sausages were tested organoleptically without any adverse effects compared with a control sausage without enzyme treatment. The structure of the enzyme treated sausages were evaluated organoleptically positive compared with the control sausage unpublished report of Danisco personnel) . The mayonnaise made using KLM3' looked similar to mayonnaise made with untreated egg yolk and was comparable in taste (unpublished report of Danisco personnel).

In the GRAS notice, we indicated that the enzyme preparation will be added to processed meats at 300LATU/kg food equal to 22.6 mg TOS/kg food and to eggs for mayonnaise

production at a maximum of 5000 LATU/kg food equal to 376.9 mg TOS/kg food . In the attached study on liver sausage, the enzyme was added from 340 LATU/kg to 790 LATU/kg food equal to 25.6-59.5 mg TOS/kg food. In practice, egg yolk for mayonnaise will be treated at 2500 LATU/kg maximum equal to 188.5 mg TOS/kg food; however, 500 to 1000 LATU/g will be the normal working dosage level. In practice, the enzyme will be added at the lowest dose needed to gain the desired technical affect. We have modified the calculation of human exposure below to take into account the maximum levels suggested from the enclosed studies.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, 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

7.4.2 Human Exposure Assessment

The GCAT enzyme provides benefits in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat.

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	2500	0.05	
Cheese	0.27	1000	0.27	
Processed meat	0.67	790	0.53	
TOTAL for US			**1.73**	**0.046**
Danish Population				
Oils	0.83	1000	0.83	
Eggs/bakery	0.17	500	0.09	
Eggs/mayonnaise	0.02	2500	0.05	
Cheese	0.52	1000	0.52	
Processed meat	0.70	790	0.55	
TOTAL for DK			**2.04**	**0.054**

[1] 1 LATU ml = 30.40 mg total protein/ml = 0.0267 mg total protein/ml

GENENCOR
A Danisco Division

Estimated Daily Intake of Acyltransferase BL1

The estimated intake of this GCAT 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[19]. The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007).

The maximum estimated daily intake of the enzyme for the US and Danish population is 0.046 and 0.054 mg total protein/kg bw/day, respectively, and these estimates represent a worst case scenario.

The maximum estimated daily intake of the enzyme from degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products is 0.054 mg total protein/kg bw/day under the scenario that (1) all above commodities are treated with the enzyme, (2) 100% of the enzyme remains in the product after processing, and (3) all consumers eat all these commodities treated with the enzyme. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing (see discussion in 6.2).

Maximum Estimated Daily Intake of the GCAT enzyme = 0.054 mg/kg bw

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, in cheese and in processed meat products (i.e., cumulative risk), the NOAEL of 41 mg total protein/kg bw/day still offers an 759X fold margin of safety and the enzyme preparation is safe for these intended uses.

This recalculation does not alter our conclusion that the KLM3' enzyme is safe for these intended uses and is GRAS. We hope that these studies and this letter have adequately answered the questions posed, if not please contact me at email: alice.caddow@danisco.com, phone: 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b)(6)

for Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures

3-23-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Vincent Sewalt [vincent.sewalt@danisco.com]
Sent: Monday, March 23, 2009 2:05 PM
To: West-Barnette, Shayla
Cc: Alice Caddow
Subject: RE: GRN 265
Attachments: GRN 265 clarification letter 032309.PDF

Dr. Barnett,

I have corrected this in the attached letter, also added to the letter that this is how it was referred to on page 30 of GRN 265. (BL stands for *Bacillus licheniformis*)

Thanks for catching this! In future GRNs we will strive to be more consistent in naming the enzyme.

Please note that I had not yet mailed the signed original to you, so by postal service you will only receive this final version.

Best regards,
Vince Sewalt

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"West-Barnette, Shayla"
<Shayla.WestBarnette@fda.hhs.gov>

To "Vincent Sewalt" <vincent.sewalt@danisco.com>
cc
Subject RE: GRN 265

Posted date : 03/23/2009 12:37:33 PM AST

Dr. Sewalt,

the OFAS review team for GRN 265 has reviewed the letter and found that the term "Acyltransferase BL1" appears in a subtitle of the letter. Could you please correct this and resend? Thank you so much and let me know if I can be of further assistance.

Shayla

From: Vincent Sewalt [mailto:vincent.sewalt@danisco.com]
Sent: Friday, March 20, 2009 4:14 PM
To: West-Barnette, Shayla
Cc: Alice Caddow
Subject: Re: GRN 265

Dr. West-Barnette,

I have added the clarification you requested to Ms Caddow's original response letter, which I have attached here as a pdf file. Do I send the original by courier to you or to Dr. Martin ? I assume just the letter will be sufficient, or do we need to resubmit other portions of the dossier?

Best regards,

Vincent Sewalt, PhD.
Director, Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Office (650) 846-5861
Mobile (650) 799-0871

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"West-Barnette, Shayla" <Shayla.WestBarnette@fda.hhs.gov>

To <vincent.sewalt@danisco.com>

Posted date : 03/19/2009 10:31:04 AM AST

cc

Subject GRN 265

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Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (office)
Shayla.WestBarnette@fda.hhs.gov

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March 23, 2009

Dr. Robert Martin
Office of Food Additive Safety, HFS-255
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

RE: GRN 000265

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We are writing in response to questions posed by FSIS and OFAS on the above GRAS Notice which were conveyed by phone conference with several OFAS staff, and email from Dr. Shayla West-Barnette, Consumer Safety Officer on March 12, 2009, and subsequent phone call and emails from Dr. West-Barnett to Dr. Vincent Sewalt on March 19 and 23.

In response to Dr. West-Barnett's request to Dr. Sewalt, we wish to clarify that in several sections of the submission the GCAT (Glycerophospholipid Cholesterol Acyltransferase) enzyme is also referred to as KLM3', our pre-launch internal code name for this enzyme. In addition, the GCAT enzyme is also referred to as Acyltransferase BL1 (on page 30 of the submission). We apologize for the inconsistency in referring to the same enzyme by three different names in this submission, but trust that this clarification suffices.

In response to the earlier request to clarify how the enzyme will be used to treat eggs, we offer the following. The action of the enzyme will be on egg yolk. For use of the egg yolk in applications such as mayonnaise, the eggs will we treated at an egg processing plant. After being separated from the egg whites, the yolks will be treated with the enzyme and pasteurized at 80° C or greater which inactivates the enzyme. They can then be sold as treated liquid yolk that is refrigerated until use or are spray dried into powder. For the baking application, the eggs will be treated by the bakery as whole eggs, but the intended effect is only on the yolk.

We have also included two studies conducted by Genencor, one on the treatment of liver sausage with KLM3' and the other on the treatment of egg yolk. Both studies demonstrate that the enzyme is effective at emulsifying the food substrate. The enzyme treated lever sausages were tested organoleptically without any adverse effects compared with a control sausage without enzyme treatment. The structure of the enzyme treated sausages were evaluated organoleptically positive compared with the control sausage unpublished report of Danisco personnel) . The mayonnaise made using KLM3' looked similar to mayonnaise made with untreated egg yolk and was comparable in taste (unpublished report of Danisco personnel).

In the GRAS notice, we indicated that the enzyme preparation will be added to processed meats at 300LATU/kg food equal to 22.6 mg TOS/kg food and to eggs for mayonnaise

production at a maximum of 5000 LATU/kg food equal to 376.9 mg TOS/kg food . In the attached study on liver sausage, the enzyme was added from 340 LATU/kg to 790 LATU/kg food equal to 25.6-59.5 mg TOS/kg food. In practice, egg yolk for mayonnaise will be treated at 2500 LATU/kg maximum equal to 188.5 mg TOS/kg food; however, 500 to 1000 LATU/g will be the normal working dosage level. In practice, the enzyme will be added at the lowest dose needed to gain the desired technical affect. We have modified the calculation of human exposure below to take into account the maximum levels suggested from the enclosed studies.

7.4.1 Identification of the NOAEL

In the 90-day oral (gavage) study in rats, 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

7.4.2 Human Exposure Assessment

The GCAT enzyme provides benefits in the degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat.

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	2500	0.05	
Cheese	0.27	1000	0.27	
Processed meat	0.67	790	0.53	
TOTAL for US			**1.73**	**0.046**
Danish Population				
Oils	0.83	1000	0.83	
Eggs/bakery	0.17	500	0.09	
Eggs/mayonnaise	0.02	2500	0.05	
Cheese	0.52	1000	0.52	
Processed meat	0.70	790	0.55	
TOTAL for DK			**2.04**	**0.054**

[1] 1 LATU ml = 30.40 mg total protein/ml = 0.0267 mg total protein/ml

GENENCOR

Estimated Daily Intake of the GCAT enzyme

The estimated intake of this GCAT 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[19]. The estimated daily intake of processed meat products was based on data from Package Food: Euromonitor from trade sources/national statistics (2007).

The maximum estimated daily intake of the enzyme for the US and Danish population is 0.046 and 0.054 mg total protein/kg bw/day, respectively, and these estimates represent a worst case scenario.

The maximum estimated daily intake of the enzyme from degumming of oil, in egg yolk and whole eggs for cakes and mayonnaise, in cheese and in processed meat products is 0.054 mg total protein/kg bw/day under the scenario that (1) all above commodities are treated with the enzyme, (2) 100% of the enzyme remains in the product after processing, and (3) all consumers eat all these commodities treated with the enzyme. In reality, it is expected that residues of a processing aid in the final products would be negligible after processing (see discussion in 6.2).

Maximum Estimated Daily Intake of the GCAT enzyme = 0.054 mg/kg bw

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, in cheese and in processed meat products (i.e., cumulative risk), the NOAEL of 41 mg total protein/kg bw/day still offers an 759X fold margin of safety and the enzyme preparation is safe for these intended uses.

This recalculation does not alter our conclusion that the KLM3' enzyme is safe for these intended uses and is GRAS. We hope that these studies and this letter have adequately answered the questions posed, if not please contact me at email: alice.caddow@danisco.com, phone: 650-846-7557 or fax at 650-845-6505.

Sincerely,

(b)(6)

for Alice J. Caddow
Vice President of Regulatory
And Environmental Affairs

Enclosures

5-4-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Alice Caddow [alice.caddow@danisco.com]
Sent: Monday, May 04, 2009 12:01 PM
To: West-Barnette, Shayla
Subject: RE: GRN 265

Hello Dr. West Barnette,

Here is the answer to your question on LATU. LATU is an abbreviation for 'Lipid Acyl Transferase Unit', the unit of acitvity for GCAT (Glycerophospholipid Cholesterol Acyltransferase) enzyme, also referred to as KLM3'.

If you should have further questions, please do not hesitate to contact me.

Sincerely,

Alice
Alice J. Caddow
Vice President of Regulatory
and Environmental Affairs
Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
Phone: 1-650-846-7557
Fax: 1-650-845-6505
email: alice.caddow@danisco.com

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5-12-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Alice Caddow [alice.caddow@danisco.com]
Sent: Tuesday, May 12, 2009 12:36 PM
To: West-Barnette, Shayla
Cc: Mcmahon, Carrie; Glew, Jeanette G
Subject: Re: Clarification of Intended Uses of GCAT Enzyme Preparation

Ms. West-Barnette,

The GRAS notice is intended to cover addition to cheese, and not other milk products. In the future, we intend to extend the uses through our GRAS panel to use GCAT in UHT milk, powdered milk and yogurt, but these uses weren't included in the original GRAS determination and the GRAS panel has not reviewed them yet, so we aren't asking for them to be included in the FDA's current review.

If you have further questions on this, please let me know. I am in the office today, but traveling for the rest of the week.

Alice J. Caddow
Vice President of Regulatory
and Environmental Affairs
Genencor, A Danisco Division
925 Page Mill Road
Palo Alto, CA 94304-1013
Phone: 1-650-846-7557
Fax: 1-650-845-6505
email: alice.caddow@danisco.com

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"West-Barnette, Shayla"
<Shayla.WestBarnette@fda.hhs.gov>

To "Alice Caddow" <alice.caddow@danisco.com>
cc "Glew, Jeanette G" <Jeanette.Glew@fda.hhs.gov>, "Mcmahon, Carrie" <Carrie.McMahon@fda.hhs.gov>
Subject Clarification of Intended Uses of GCAT Enzyme Preparation

Posted date : 05/12/2009 12:26:17 PM AST

Ms Caddow,

In working to complete our evaluation of GRN 265 (subject: GCAT Enzyme Preparation), we have an additional point for which we need clarification. On pages 3, 4 and 14 of the notice, you state that one of the intended uses of GCAT is in "milk products like cheese". However, the only milk product discussed in the notice is cheese. Do

you intend to add GCAT enzyme preparation to other milk products? If so, could you give examples of what those additional milk products are? It may be helpful to refer to Part 170.30(1)(n) of Title 21 of the Code of Federal Regulations (21 CFR 170.30(1)(n)) to understand how foods are grouped according to FDA. The regulation can be viewed at http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=9f3d58edbdc5a070c323561f82ab9f60&rgn=div8&view=text&node=21:3.0.1.1.1.1.1.1&idno=21, and lists the general food categories that are established to group specific related foods together for the purpose of establishing tolerances or limitations for the use of direct human food ingredients. Note that cheese and milk products are listed as separate food categories; cheese is listed under (21 CFR 170.30(1)(n)(5), and milk products are listed under 21 CFR 170.30(1)(n)(31).

A response by close of business (your time) Wednesday would be greatly appreciated. If you will need additional time to respond to these questions, or if you have questions regarding this matter, please do not hesitate to contact me. Thanks,

Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (office)
Shayla.WestBarnette@fda.hhs.gov

6-30-09 Correspondence from Notifier

AM

West-Barnette, Shayla

From: Vincent Sewalt [vincent.sewalt@danisco.com]
Sent: Tuesday, June 30, 2009 11:30 AM
To: West-Barnette, Shayla
Cc: Alice Caddow
Subject: Fw: Regarding Your Concern About FDA's Response to GRN 265

Dear Dr. Barnette,

After discussing the issue with Alice Caddow, we concluded that the information in the original GRAS submissions (GRN 265 and GRN 277) is not considered confidential as such, so when the time comes for those submissions to be posted on-line that can be done without any alterations. My apologies for the misunderstanding, which resulted from my conservative approach in absence of Alice.

However, my request to omit the specific amino acid substitutions in your response letter still stands as your response letter will be posted on-line immediately and will reach a wider audience. Hence, as previously indicated we request to combine the last two sentences of the first full paragraph on page 2 of the Agency's response letter to GRN 265 as follows:

"Genencor notes that the amino acid sequence of the GCAT enzyme has been modified from that of the native GCAT enzyme from *A. salmoninicida*subsp. *salmonicida*in only one amino acid, and that this modification optimizes GCAT expression in *B. licheniformis.*"

Once an updated letter is available, could you please email it to us ?

Thanks for your cooperation.

Best regards,

Vincent Sewalt, PhD
Director, Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Office (650) 846-5861
Mobile (650) 799-0871

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----- Forwarded by Vincent Sewalt/Palo Alto/Danisco on 06/30/2009 08:28 AM -----

Vincent Sewalt/Palo Alto/Danisco

06/22/2009 01:28 AM

To "West-Barnette, Shayla" <Shayla.WestBarnette@fda.hhs.gov>
cc Alice Caddow/Palo Alto/Danisco@Danisco
Subject Re. Regarding Your Concern About FDA's Response to GRN 265 Link

Dear Dr. West-Barnette,

Thank you for your response below. We appreciate your efforts not to post the response letter to GRN 265 online in its current form, and for the opportunity to suggest alternative language that will resolve Danisco's concern while keeping intact the safety information that forms the basis for the GRAS determination. We suggest the following alternative language that combines the last two sentences of the first full paragraph on page 2 of the Agency's response letter to GRN 265:

"Genencor notes that the amino acid sequence of the GCAT enzyme has been modified from that of the native GCAT enzyme from *A. salmoninicida*subsp. *salmonicida*in only one amino acid, and that this modification optimizes GCAT expression in *B. licheniformis.*"

I understand that we indeed had not indicated in our submission that any of the information was confidential to Danisco. This is because of our understanding that the specific mutations in protein-modified enzymes were not being disclosed in the Agency's response letters. We verified this in the GRNs known to us as pertaining to protein-modified enzymes, and this is certainly the case. We did notice that, indeed, your office is currently in the process of publishing the full GRAS Notices on your website. As Danisco does consider the specific amino acid substitution in GCAT to be confidential information, will it be possible to have the Agency omit that information from GRN 265? In the confidentially notice you are referring to below to indicate the confidential information, do you need Danisco to indicate the pages and lines containing the specific amino acid substitution information or do you need us to submit a version of GRN 265 with that information marked or deleted ? Please let me know (with cc to Ms. Alice Caddow) so we can take appropriate action.

In addition to GRN 265, Danisco recently submitted another Notice (GRN 277) for a protein-engineered enzyme (G4 amylase). In the Agency's response to GRN 277 the specific amino acid changes were not disclosed, so that response letter is fine. However, coming to the realization that the full GRAS Notice will be published on your website, disclosure of the specific amino acid substitutions in the G4 amylase become a concern as well. I have not discussed this with Ms. Alice Caddow as she is out of the office, but I suspect that Danisco would strongly prefer not to disclose this information either. As GRN 277 and its response letter have not been posted on CFSAN's web site yet either, could you please request that GRN 277 will not be posted until our concerns around the confidential information in that GRAS Notice is resolved as well?

Again, we appreciate your understanding and assistance to Danisco sofar. If you could please copy Ms, Caddow on your reply, she will be able to action sending you a confidentiality notice for GRN 265 (indicating the exact confidential information in GRN 265 either in the letter or in the original GRN) and also any follow-up for GRN 277.

Best regards,

Vincent Sewalt, PhD
Director, Regulatory Affairs
Genencor, a Danisco Division
925 Page Mill Road
Palo Alto, CA 94304
Office (650) 846-5861

Mobile (650) 799-0871

"West-Barnette, Shayla"
<Shayla.WestBarnette@fda.hhs.gov>

To "Vincent Sewalt" <vincent.sewalt@danisco.com>

cc "Alice Caddow" <alice caddow@danisco.com>

Subject Regarding Your Concern About FDA's Response to GRN 265

Posted date : 06/19/2009 11:46:45 AM AST

Dr. Sewalt,

Thank you for communicating with me by phone message on June 15, 2009 and by telephone on June 16, 2009 regarding Danisco's concern about FDA's response letter, dated June 15, 2009, to GRN 265 (subject: GCAT enzyme preparation). Specifically, you expressed concern over the level of detail in FDA's response letter about an amino acid change that Danisco engineered in the GCAT enzyme. To my understanding, this text is found at the end of the first full paragraph on page 2 of the letter. Given Danisco's concern, can you provide a specific recommendation for revision to the letter that would address this concern ? Please keep in mind that the GRAS use of a substance is based on general recognition and public availability of the safety information upon which a GRAS determination is based.

Danisco's GRAS notice did not indicate that any information or detail therein was considered by the notifier to be confidential. Our office is in the process of making all GRAS notices, as well as FDA's responses to these notices, available on our website. For your information, I have ensured that GRN 265 and FDA's response to the notice will not be posted until we resolve your concerns.

I also remind you that GRAS notices are releasable under the Freedom of Information Act (FOIA). FDA responded to a FOIA request for a copy of Danisco's notice on February 4, 2009. Is it Danisco's opinion that the level of detail regarding the amino acid change included in GRN 265 constitutes confidential commercial information that should be exempt from FOIA release? If so, please provide a copy of Danisco's notice which indicates the information considered by Danisco to be confidential. Again, please keep in mind that the GRAS use of a substance is based on general recognition and public availability of the safety information upon which a GRAS determination is based.

I look forward to receiving information from you about a recommendation for revision to FDA's response letter, as well as whether Danisco considers the information about the amino acid change in GRN 265 confidential. If you have further questions, please feel free to contact me.

Regards,
Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (office)
Shayla.WestBarnette@fda.hhs.gov