SCIENTIFIC OPINION

Scientific Opinion on Lipase from a Genetically Modified Strain of *Aspergillus oryzae* (strain NZYM-LH)[1,2]

EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids (CEF)[3,4]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The food enzyme considered in this opinion is a lipase (triacylglycerol lipase; EC 3.1.1.3) produced with a genetically modified strain of *Aspergillus oryzae*. The genetic modifications do not raise safety concern. The food enzyme contains neither the production organism nor recombinant DNA. The lipase is intended to be used in a number of food manufacturing processes, such as in baking and other cereal-based processes. The dietary exposure was assessed on the basis of data retrieved from the EFSA Comprehensive European Food Consumption Database. The food enzyme did not induce gene mutations in bacteria nor micronuclei in human peripheral blood lymphocytes. Therefore, there is no concern with respect to genotoxicity. The systemic toxicity was assessed by means of a 90-day subchronic oral toxicity study in rodents. A No Observed Adverse Effect Level was derived, which compared with the dietary exposure results in a sufficiently high Margin of Exposure. The allergenicity was evaluated by searching for similarity of the amino acid sequence to those of known allergens. The Panel considered that the likelihood of food allergic reactions to the enzyme is low and therefore does not raise safety concern. Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided and the toxicological studies, this food enzyme does not raise safety concern under the intended conditions of use.

KEY WORDS

food enzyme, lipase, triacylglycerol lipase, EC 3.1.1.3, *Aspergillus oryzae*, genetically modified microorganism

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[1] On request from the European Commission, Question No EFSA-Q-2012-01009, adopted on 20 June 2014.

[2] This scientific opinion replaces the previously published summary of the opinion following the provisions of article 12(3) of Regulation (EC) No 1331/2008.

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Food Contact Material, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the food enzyme lipase (triacylglycerol lipase; EC 3.1.1.3) produced with the genetically modified *Aspergillus oryzae* strain NZYM-LH.

The *A. oryzae* parental strain has a long history of use for the production of food enzymes. It has been modified in order to produce and secrete lipase and to prevent or decrease the production of undesirable secondary metabolites. The genetic modifications do not raise safety concern.

The food enzyme contains neither the production organism nor recombinant DNA, given the limits of detection.

The food enzyme has been characterised by determining the temperature and pH optima and the thermo-stability. Its composition is verified by measuring the content of protein, ash, water, heavy metals and Total Organic Solids. The *A. oryzae* parental strain has been modified in order to prevent the production of cyclopiazonic acid and to decrease the potential production of kojic acid. The absence of cyclopiazonic acid, β-nitropropionic acid and kojic acid was demonstrated, given the limits of detection.

The food enzyme is intended to be used in a number food manufacturing processes, such as in baking and other cereal-based processes. The typical use and the use levels recommended for specific food processes have been provided.

The estimated dietary exposure was calculated on the basis of data retrieved from the EFSA Comprehensive European Food Consumption Database.

The genotoxicity of the food enzyme was assessed by means of two *in vitro* assays (gene mutations in bacteria and *in vitro* micronucleus assay). The food enzyme, produced with the genetically modified *A. oryzae* strain NZYM-LH, did not induce gene mutations in bacteria with or without metabolic activation when tested under the conditions employed in the study as presented by the applicant. Neither did it induce micronuclei in cultured human blood lymphocytes under the test conditions employed for this study. The systemic toxicity was assessed by means of a 90-day subchronic oral toxicity study in rodents. A No Observed Adverse Effect Level (NOAEL) was derived, which compared with the dietary exposure results in a sufficiently high Margin of Exposure.

The CEF Panel considers that the likelihood of food allergic reactions to this lipase produced with this genetically modified strain of *A. oryzae* is low and therefore does not raise safety concern.

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided and the toxicological studies, the Panel concluded that this food enzyme does not raise safety concern under the intended conditions of use.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Only food enzymes included in the Union list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008[5] on food enzymes. According to Regulation (EC) No 1332/2008 on food enzymes, a food enzyme which falls within the scope of Regulation (EC) No 1829/2003[6] on genetically modified food and feed should be authorised in accordance with that Regulation as well as under this Regulation.

An application has been introduced by the company Novozymes A/S for the authorisation of the food enzyme lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-LH).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[7] implementing Regulation (EC) No 1331/2008[8], the Commission has verified that the application falls within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to carry out the safety assessment on the food enzyme lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-LH) in accordance with the article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

[5] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/199, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, p. 7-15.

[6] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003 p. 1-23.

[7] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.03.2011, p 15-24.

[8] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p 1-6.

ASSESSMENT

1. Introduction

Before January 2009 food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009 the Regulation (EC) No 1332/2008 on food enzymes entered into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. The Regulation (EC) No 1331/2008 established Union procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall only be authorised if it is demonstrated that:

(i) it does not pose a safety concern to the health of the consumer at the level of use proposed;

(ii) there is a reasonable technological need, and

(iii) its use does not mislead the consumer.

All food enzymes currently on the EU market and intended to remain on the market as well as all new food enzymes shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and an approval via a Union list.

The Guidance on submission of a dossier on a food enzyme for evaluation by EFSA (EFSA, 2009) lays down the administrative, technical and toxicological data required.

In the case of enzymes produced with genetically modified microorganisms (GMM) the guidance on the risk assessment of GMM and their products intended for food and feed use (EFSA GMO Panel, 2011) applies.

The assessment of a GMM aims to evaluate the safety of the food enzyme related to the genetic modifications. This starts from the evaluation of the safety of the parental/recipient and donor strains, e.g. regarding their capability to produce undesirable metabolites. It also includes the assessment of the genetic modifications, specifically of the introduction of genes of concern and the genetic stability. Finally, the presence of the production organism and its recombinant DNA in the final product is assessed. Special attention is paid to the presence of any recombinant genes of concern (e.g. antibiotic resistance genes), introduced during the development of the production strain. If the absence of the production strain and the recombinant DNA has been confirmed, no extensive environmental risk assessment of the enzyme product is required.

The following evaluation applies to the lipase (triacylglycerol lipase; EC 3.1.1.3) produced with a genetically modified *A. oryzae* strain NZYM-LH.

2. Information on existing authorisations and evaluations

The applicant reports that the Danish and French authorities have evaluated and authorised the use of the food enzyme produced with the genetically modified *A. oryzae*, strain NZYM-LH for baking processes. The Danish authority also provided the conditions of use, including the dosages for specific foods that were up to a level of 400 LU[9]/kg flour.

[9] LU = Lipase Units (see Section 3.3)

3. Technical data

3.1. Identity of the food enzyme

IUBMB nomenclature: Triacylglycerol lipase

Systematic name: Triacylglycerol acylhydrolase

Synonym: Lipase

IUBMB No: EC 3.1.1.3

CAS No: 9001-62-1

EINECS No: 232-619-9.

3.2. Chemical parameters

The lipase produced with the genetically modified *A. oryzae* strain NZYM-LH is a single polypeptide chain of 317 amino acids. The molecular mass derived from the amino acid sequence was calculated to be 35.0 kDa.

Data on the chemical parameters and the protein homogeneity status of the food enzyme have been provided for four food enzyme batches, three batches to be used for commercialisation and one batch (PPW 26090) used for the toxicological tests (Table 1).

The average Total Organic Solids (TOS) of the four food enzyme batches was 9.7 % (w/w); the values ranged from 9.1 to 10.3 % (Table 1). TOS is a calculated value derived from 100 % minus % water minus % ash. The four food enzyme batches presented in Table 1 are concentrates without any added diluents.

The average specific activity of the four food enzyme batches expressed as ratio enzyme activity/mg TOS was 0.108 KLU/mg TOS; the values ranged from 0.095 to 0.116 KLU/mg TOS (Table 1). Considering the low variability of the activities as well as the specific activities in the four food enzyme batches, the average activity/mg TOS value of 0.108 KLU/mg TOS was used for subsequent calculations.

A. oryzae, as a species, is known to have the potential to produce undesirable secondary metabolites such as cyclopiazonic acid (CPA), β-nitropropionic acid (NPA) and kojic acid (KA) (Blumenthal, 2004). Accordingly, these mycotoxins must be checked in the final food enzyme from *A. oryzae* (FAO/WHO, 2006). *A. oryzae* as a species belongs to the *Aspergillus flavus* group which is known to have a gene cascade for the biosynthesis of aflatoxins, but under any known fermentation conditions, *A. oryzae* strains never produced aflatoxins (Blumenthal, 2004; Lee et al., 1991). The parental strain has been modified by γ-irradiation mutagenesis, resulting in the deletion of gene clusters required for the synthesis of the mycotoxins cyclopiazonic acid (*cpa*) and aflatoxin (*afl*). UV irradiation mutagenesis resulted in a reduced potential (15 % of the original) to produce kojic acid. None of these three species-specific mycotoxins (CPA, NPA and KA) were detected in the food enzyme (Table 1) by mass spectrometry (LC-MS/MS).

The protein homogeneity status of the food enzyme was also investigated by SDS-PAGE analysis. The apparent molecular mass based on this technique is about 36 kDa. The gels presented for the four food enzyme batches consistently showed a protein band corresponding to a molecular mass of about 36 kDa.

The food enzyme was tested for other enzyme activities, i.e. alpha-amylase, glucoamylase (amyloglucosidase) and protease activities, which were below the detection limits of the employed methods (Table 1).

Table 1: Compositional data of the food enzyme

Parameter	Unit	Batches			
		LGF 0029	LGF 0030	LGF 0031	PPW 26090[a]
Lipase activity	KLU/g batch[b]	11.3	10.0	9.2	11.3
Protein	% (w/w)	5.94	5.63	4.88	5.94
Ash	% (w/w)	1.6	1.5	1.3	1.6
Water	% (w/w)	88.7	89.4	89.0	88.1
Total Organic Solids (TOS)[c]	% (w/w)	9.7	9.1	9.7	10.3
Activity/mg TOS	KLU/mg TOS	0.116	0.110	0.095	0.110
Pb	mg/kg batch	ND[d]	ND[d]	ND[d]	ND[d]
As	mg/kg batch	ND[d]	0.115	0.136	ND[d]
Cd	mg/kg batch	ND[d]	ND[d]	ND[d]	ND[d]
Hg	mg/kg batch	ND[d]	ND[d]	ND[d]	ND[d]
Antimicrobial activity		ND[d]	ND[d]	ND[d]	ND[d]
Production strain	CFU per g batch	NA[e]	NA[e]	NA[e]	ND[d]
Cyclopiazonic acid	mg/kg batch	ND[d]	ND[d]	ND[d]	ND[d]
Kojic acid	mg/kg batch	ND[d]	ND[d]	ND[d]	ND[d]
β-nitro propionic acid	mg/kg batch	ND[d]	ND[d]	ND[d]	ND[d]
Antifoam agents	mg/kg batch	393	354	110	398
Alpha-amylase	FAU(F)/g batch[f]	ND[d]	ND[d]	ND[d]	ND[d]
Glucoamylase	AGU/g batch[g]	ND[d]	ND[d]	ND[d]	ND[d]
Protease	HUT/g batch[h]	ND[d]	ND[d]	ND[d]	ND[d]

(a): Batch used for the toxicological tests.
(b): KLU: Kilo Lipase Units (see Section 3.3).
(c): TOS calculated as 100 % - % water -% ash.
(d): ND: Not detected: below the limits of detections (Pb: 0.5 mg/kg; As: 0.1 mg/kg; Cd: 0.05 mg/kg; Hg: 0.03 mg/kg; cyclopropionic acid: 0.100 mg/kg; kojic acid: the limits of detection ranged from 0.03 to 0.08 mg/kg; β-nitropropionic acid: 0.23 mg/kg for three batches, 0.20 mg/kg for batch LGF 0031; alpha-amylase: 0.34 FAU(F)/g; glucoamylase: 0.825 AGU/g: protease: 196 HUT/g; antimicrobial activity: inhibition zone diameters ≤ 16 mm imply that the antimicrobial activity was absent (FAO/WHO, 2006)).
(e): NA: Not analysed.
(f): FAU(F): Fungal alpha-Amylase Units (relative to an internal enzyme standard "F").
(g): AGU: AmyloGlucosidase Units.
(h): HUT: Hemoglobin Units Tyrosine.

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms are not more than 30 CFU (Colony Forming Units) per gram.

The applicant has provided information on the identity of the antifoam agents and the Panel considers the use of these as of no safety concern.

The provided data regarding compositional batch-to-batch-variability are considered sufficient. Table 1 shows that the food enzyme batch PPW 26090 used for the toxicological assays has a similar

activity/mg TOS, level of inorganic constituents and amount of antifoam agents in relation to the other three food enzyme batches (LGF). Consequently, this food enzyme batch (PPW 26090) is considered suitable for the toxicological testing.

3.3. Properties of the food enzyme

Triacylglycerol lipases catalyse the hydrolysis of the ester linkages in triacylglycerols, thus resulting in the generation of free fatty acids, diacylglycerols, monoacylglycerols and glycerol. It also hydrolyses the sn-1-ester linkage of diacylphospholipids resulting in the formation of 2-acyl-1-lysophospholipid and free fatty acid. It does not require any co-factor.

The enzymatic activity is quantified by using a pH-stat titration system and is expressed in Kilo Lipase Units/g (KLU/g). One LU is the amount of enzyme which releases 1 µmol of titratable butyric acid per minute under the given standard conditions (reaction conditions: pH = 7.0, T = 30 °C, reaction time, at least 1.5 minutes). One LU corresponds to the international definition of an enzyme unit. In the assay tributyrin is used as substrate. Its cleavage results in butyric acid formation, which cause a pH variation. The reaction rate, and therefore the enzyme activity, are determined by measuring the volume of titrant added to the reaction system per minute to keep the pH constant.

The food enzyme has been characterised regarding its activity depending on temperature and pH. The lipase is active at temperatures up to 70 °C (with an optimum at approximately 40 °C at pH 6) and within the pH-range of 4 to 10 (with an optimum of about pH 8 at 30 °C). The thermo-stability of the lipase was tested over the range of 30 °C to 90 °C after a pre-incubation at the different temperatures at pH 6 for 30 minutes. The activity itself was measured under standard conditions. The food enzyme retained its activity at temperatures up to 50 °C. At higher temperatures, the food enzyme losses rapidly its activity (at 60 °C, 40 % residual activity, pre-incubation at pH 6 for 30 minutes) and above 80 °C, no activity remains after 30 minutes.

3.4. Information on the source materials

3.4.1. Information relating to the genetically modified microorganism

3.4.1.1. Characteristics of the recipient and parental microorganisms

The production organism is the fungus *A.oryzae*. *A. oryzae* strains are not qualified as QPS (Qualified Presumption of Safety) because of the potential of mycotoxin production (EFSA BIOHAZ, 2012). The parental strain, *A. oryzae* A1560, has a long history of use for the production of food enzymes. The recipient strain *A. oryzae* JaL830, has been developed from the parental strain, *A. oryzae* A1560, through a series of modification steps including classical mutagenesis and genetic modification (the latter not described for confidentiality reasons).

The classical mutagenesis steps included γ-irradiation mutagenesis, resulting in the deletion of gene clusters required for the synthesis of the mycotoxins cyclopiazonic acid (*cpa*) and aflatoxin (*afl*), and UV irradiation mutagenesis, resulting in a drastically reduced potential (15 % of the original) to produce kojic acid. A Southern blot analysis confirmed the absence of antibiotic resistance genes which were used during the genetic modification of the recipient strain.

3.4.1.2. Characteristics of the donor organisms

The plasmid vector used for the transformation of the recipient strain contained elements to control the expression of the gene of interest as well as ensure integration of the introduced DNA into the recipient strain chromosome. The DNA introduced into the recipient strain did not contain antibiotic resistance genes.

3.4.1.3. Description of the genetic modification process

The production strain *A. oryzae* NZYM-LH was developed from the recipient strain JaL830 through transformation with a plasmid vector and selection of the transformants on appropriate medium.

The production strain differs from the recipient strain by the synthesis and secretion of lipase.

3.4.1.4. Safety aspects of the genetic modification: Information relating to the GMM and comparison of the GMM with its conventional counterpart

In comparison to the parental strain A1560, the recipient strain has lost the potential to produce cyclopiazonic acid and has a reduced potential to produce kojic acid.

The final production strain NZYM-LH differs from the recipient strain by the synthesis and secretion of lipase.

The presence of the gene encoding lipase in the NZYM-LH strain was verified by Southern blot analysis; this indicated that multiple copies of the full-length gene were integrated in the chromosomal DNA of the recipient. In order to estimate the number of copies, a quantitative real-time PCR was carried out.

Southern blot analysis of the DNA production strain from three independent enzyme preparation batches at the end of pilot scale fermentation confirmed the genetic stability of the genetic modifications.

The genetic modifications do not raise safety concern.

3.4.1.5. Safety for the environment

Neither the production strain nor its recombinant DNA were detected in the final product (see Section 3.5.2). Accordingly, as the food enzyme belongs to Category 2 of the guidance on risk assessment of genetically modified microorganisms and their products (EFSA GMO Panel, 2011), environmental exposure to the genetically modified microorganism or its DNA is negligible and hence no further environmental risk assessment is required.

3.5. Manufacturing process

The manufacturing process includes a fermentation process and downstream processing. A comprehensive dataset related to the manufacturing process including a flow diagram was provided. The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[10]. According to the applicant the manufacturing process is certified according to Food Safety Systems Certification 22000 (FSSC 22000) and ISO 9001.

3.5.1. Information relating to the fermentation process

The food enzyme is produced by a pure culture in contained submerged fed-batch fermentation with conventional process controls in place. The identity and purity of the culture are checked at each transfer step from frozen vials to the end of fermentation.

3.5.2. Information relating to the downstream processing

The downstream processing includes recovery, purification and concentration. The food enzyme produced is recovered from the fermentation broth after biomass separation via filtration. Further purification and concentration involve a series of filtration steps, including ultrafiltration and final germ filtration.

[10] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, p. 3-21.

The production strain could not be detected in a test volume of 1 g of three independent liquid batches tested in triplicate by liquid culturing in non-selective medium for 4 days (for resuscitation) followed by growth on selective solid agar plates for 4 days at suitable temperature favouring the growth of the production strain. No recombinant DNA was detected starting with 1 g of three samples of the concentrated product before formulation obtained from three independent production batches and tested in triplicate. Analysis was performed by PCR, amplifying the recombinant fragment spanning the deletion of an endogenous gene specific for the strain lineage, introduced in the first genetic modification step of the parental strain.

3.6. Reaction and fate in food

Triacylglycerol lipases catalyse the hydrolysis of the ester linkages in triacylglycerols. The hydrolysis of triacylglycerols results in the generation of free fatty acids, diacylglycerols, monoacylglycerols and glycerol. It also hydrolyses the sn-1-ester linkage of diacylphospholipids resulting in the formation of 2-acyl-1-lysophospholipid and free fatty acid.

The data and information provided indicate that the lipase is denatured during processing under the intended use conditions. The food enzyme was tested for other enzyme activities i.e. alpha-amylase, glucoamylase (amyloglucosidase) and protease activities, which were below the detection limits of the employed methods. Therefore, no unintended reaction products from the lipase and any side-activities are to be expected.

3.7. Case of need and intended conditions of use

The food enzyme is intended to be used for baking and other cereals processes (Table 2).

Table 2: Typical uses and recommended maximum use levels of the food enzyme as provided by the applicant

Process	Recommended dosage of the food enzyme
Baking processes and other cereal-based processes	Up to 2.2 KLU/kg of flour or grain, corresponding to 20.4 mg TOS/kg (2.2 KLU/0.108 KLU/mg TOS (0.108 is the average of the four batches))

In the baking process, the use of lipase is aimed to improve the characteristic of the dough (strength and stability) facilitating its handling. The food enzyme is added together with other raw materials during the dough formation step.

In other cereal-based processes, like processing of products such as pasta, noodles and snacks, the use of lipase is aimed to improve the dough processability, to reduce oil uptake during frying and to accelerate the drying step, thereby shortening the process time.

According to the applicant, the food enzyme is used at the minimum dosage necessary to achieve the desired reaction according to Good Manufacturing Practice. The dosage applied in practice by a food manufacturer depends on the particular process (see Table 2).

4. Dietary exposure

The dietary exposure assessment is performed for the processes for which the food enzyme is intended to be used (Table 2). A maximum consumption of baked product in 17 countries of 23.3 g/kg bw/day was retrieved from the EFSA Comprehensive European Food Consumption Database[11]. It was estimated that the baking process results (on average) in 140 g of final bakery product from 100 g of flour. Therefore, an intake of flour of 16.6 g/kg bw/day is calculated.

[11]EFSA Comprehensive European Food Consumption Database **http://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb.htm**

Taking into account the highest recommended dosage of the food enzyme, 2 200 LU per kilogram of flour, corresponding to 20.4 mg TOS, it is possible to obtain the estimated dietary exposure, which results in 0.339 mg TOS/kg bw/day (20.4 mg TOS/kg flour x 16.6 x 10^{-3} kg flour/kg bw/day).

In case the food enzyme is proposed for products particularly designed for infants or young children, *ad hoc* conservative exposure estimates must be made taking specifically into account these population groups. Otherwise, the very conservative approach described above, is considered to cover both adults and children.

5. Toxicological data

The toxicological assays were performed with a food enzyme (batch PPW 26090, see Table 1) representative of the other three food enzyme batches.

Genotoxicity testing

In order to investigate the potential to induce gene mutations in bacteria, an Ames test was performed according to OECD Test Guideline 471 (OECD, 1997) and following Good Laboratory Practice (GLP) in four strains of *Salmonella typhimurium* (TA1535, TA100, TA1537, TA98) and *Escherichia coli* WP2*uvrA*, in the presence or absence of metabolic activation by S9 mix applying the "plate incorporation assay" for the *E. coli* strain and the "treat and plate assay" for the Salmonella strains. Two experiments were carried out using six different concentrations of the food enzyme, appropriate positive control chemicals and deionised water as solvent and negative control. The highest concentration was 5 000 µg dry matter (4 300 µg TOS) per ml incubation mixture (treat and plate assay) or per plate (plate incorporation assay). All positive control chemicals induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9 mix, while the negative controls were within the normal ranges. Significant cytotoxicity, measured as the number of viable cells, was observed in one experiment with TA1537 in the presence of metabolic activation. Therefore, a further experiment was conducted with TA1537 in the presence of metabolic activation including treatments with a heat-inactivated sample of the test article. No toxicity was observed in treatments with the heat-inactivated sample in this third experiment but a concentration-dependent increase in cytotoxicity was observed for the non-heat treated sample in this experiment. There were no signs of an increase in revertants at any concentration tested for all the five test strains in both the presence and absence of metabolic activation. Therefore, it was concluded that the food enzyme has no mutagenic activity, under the conditions employed.

The *in vitro* micronucleus assay was carried out according to OECD Test Guideline 487 (OECD, 2010) and following GLP. The food enzyme was tested in the *in vitro* micronucleus test using human peripheral blood lymphocytes both with and without metabolic activation with S9 mix. A maximum concentration of 5000 µg food enzyme/ml final culture concentration (corresponding to 515 µg TOS/ml final culture concentration) was selected for the Range-Finder Experiment. Concentrations for the Main Experiment were selected based on the results of this cytotoxicity Range-Finder Experiment. Purified water was used as solvent, and appropriate positive controls were included. Scoring was done with 1 000 cells in duplicate cultures. In experiment one, the highest concentration (5 000 µg food enzyme/ml culture, corresponding to 515 µg TOS/ml culture) in the absence and presence of metabolic activation induced approximately 0 % and 21 % reduction in replication index, respectively. There were no indications of an increase in micronucleated cells at the three tested concentrations compared to the vehicle control both in the absence and presence of metabolic activation. In experiment two, the highest concentration (5 000 µg food enzyme/ml culture, corresponding to 515 µg TOS/ml culture) in the absence and presence of metabolic activation, induced approximately 0 % and 15 % reduction in replication index, respectively. There were no indications of an increase in micronucleated cells at the three tested concentrations compared to the vehicle control both in the absence and presence of metabolic activation. It was concluded that the food enzyme did not induce chromosomal aberration in cultured human peripheral blood lymphocytes when tested up to 5 000 µg food enzyme/ml (corresponding to 515 µg TOS/ml) under the experimental conditions employed.

Repeated dose toxicity testing:

A 90-day subchronic oral toxicity study was performed according to OECD Test Guideline 408 and following GLP (OECD, 1998). Four groups of 10 male and 10 female SPF Sprague Dawley rats of the Ntac:SD strain were given by gavage a dose of 10 ml/kg bw/day tap water, a 10 % solution, a 33 % solution as well an undiluted stock solution of the food enzyme for 13 weeks. The doses were equivalent to 108, 357 and 1 080 mg TOS/kg bw/day.

In open field observations it was noticed that females in the highest dose group showed statistically significant less time of moving, and also had a tendency for improper grasp response. However, these findings were isolated and of low magnitude and therefore they were not considered to be adverse.

In haematology, a statistically significant decrease in the percentage of neutrophils and an increase in the percentage of lymphocytes were found in all male groups given the enzyme but there was no dose-response between these groups. A statistically significant decrease in the number of neutrophils was measured in females in the low dose and in males in the low and medium dose groups. A significant higher percentage of eosinophils was seen in females of the medium dose group. These effects were observed sporadically, were not dose related and consequently, were not considered toxicologically significant.

Blood analysis revealed that the aspartate aminotransferase activity in high dose males, as well as the potassium level in females of the medium dose group, were statistically significant lower than those of the control group. These effects were observed sporadically and were not dose related.

Regarding the organ weight observations, the absolute brain weight in the low dose group of males only was statistically significantly lower than the control group. The observed statistically significant findings were not treatment related.

Due to an experimental error, ophthalmoscopy was not performed at the end of the study as requested in the OECD guideline. Considering the absence of histopathological findings in the eyes, the missing ophthalmoscopy was not considered to affect the conclusion of the study.

Based on the above mentioned observations, the Panel derived a NOAEL of the high dose level 1 080 mg TOS/kg bw/day.

6. Allergenicity

Potential allergenicity of lipase was assessed by comparing its amino acid sequence with those of known allergens according to the EFSA Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35 % identity in a window of 80 amino acids as the criterion, no match was found. No food allergic reactions to lipase have been reported in the literature.

Consequently, the CEF Panel considers that the likelihood of food allergic reactions to this lipase produced with this genetically modified strain of *A. oryzae* is low and therefore does not raise safety concerns.

7. Discussion

The parental strain *A. oryzae* A1560 has a long history of use for the production of food enzymes. The recipient strain, JaL830, was developed from the parental strain through a series of modification steps including classical mutagenesis and genetic modification. The production strain NZYM-LH contains a recombinant gene encoding lipase. Multiple copies were randomly integrated into the genomic DNA as part of the plasmid vector. The introduced trait is well-known and does not trigger a safety concern. No sequences that can cause safety concerns (e.g. antibiotic resistance genes) remained. Neither the production strain nor the recombinant genes are detected in the final products by methods considered adequate by the CEF Panel.

The information provided on the manufacturing of the food enzyme, i.e. the fermentation conditions and the steps employed for isolation and purification, is considered sufficient. The available compositional data, including experimental evidence of the absence of potential contaminants such as mycotoxins, sufficiently demonstrate the identity and the purity of the food enzyme. The reported batch-to-batch variability is considered acceptable.

The process conditions provided indicate that the enzyme is inactivated in the course of the intended applications.

Dietary exposure estimates were calculated by assuming that the food enzyme is used at its maximum recommended dosage and the entire added food enzyme remains in the final food. Based on these assumptions and considering the total intake (Section 4), the estimated dietary exposure results to be 0.339 mg TOS/kg bw/day.

The food enzyme produced with the genetically modified *A. oryzae* strain NZYM-LH did not induce gene mutations in bacteria with or without metabolic activation. Neither did it induce chromosome aberrations in cultured human blood lymphocytes. Therefore, there is no concern with respect to genotoxicity.

A comparison of the NOAEL (1 080 mg TOS/kg bw/day) from the 90-day study with the estimated dietary exposure, calculated to be 0.339 mg TOS/kg bw/day, results in a margin of exposure (MOE) of 3 186, which is found sufficient.

The CEF Panel considers that the likelihood of food allergic reactions to this lipase from this genetically modified strain of *A. oryzae* is low and therefore does not raise safety concerns.

CONCLUSIONS

Based on the genetic modifications performed, the manufacturing process, the compositional and biochemical data provided and findings in the toxicological studies, the food enzyme "Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-LH)" does not raise a safety concern under the intended conditions of use.

DOCUMENTATION PROVIDED TO EFSA

Dossier "Lipase from a genetically modified strain of *Aspergillus oryzae* (strain NZYM-LH)". November 2012. Submitted by Novozymes A/S.

Additional information received by Novozymes A/S on August 2013.

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ABBREVIATIONS

Afl	aflatoxin
AGU	AmyloGlucosidase Units.
bw	Body weight
CAS	Chemical Abstracts Service
CFU	Colony Forming Units
CPA	Cyclopiazonic acid
DNA	Deoxyribonucleic acid
EC	European Commission and Enzyme Commission
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
FAU	Fungal alpha-Amylase Units
FAO	Food and Agricultural Organisation
GLP	Good Laboratory Practice
GMM	Genetically Modified Micro-organisms
GMO	Genetically Modified Organisms
GMP	Good Manufacturing Practice
HUT	Hemoglobin Units Tyrosine
IUBMB	International Union of Biochemistry and Molecular Biology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KA	Kojic acid
KLU	Kilo Lipase Units
LC-MS/MS	Liquid Chromatography/Tandem Mass Spectrometry
LU	Lipase Units
MOE	Margin of Exposure
MS	Mass Spectrometry
NOAEL	No Observed Adverse Effect Level
NA	Not Analysed
ND	Not Detected
NPA	β-nitropropionic acid
OECD	Organisation for Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PS	Qualified Presumption of Safety
QPS	Qualified Presumption of Safety
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
TOS	Total Organic Solids
WHO	World Health Organisation