PHOSPHOLIPASE A1 FROM FUSARIUM VENENATUM *EXPRESSED IN* ASPERGILLUS ORYZAE

First draft prepared by

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1. *EXPLANATION*

The enzyme preparation that was evaluated contains the enzyme phospholipase A1 (phosphatidylcholine 1-acylhydrolase), which has not been evaluated previously by the Committee. Phospholipase A1 is an enzyme that acts specifically on the fatty acid in position 1 of phospholipid substrates, resulting in the formation of lysophospholipids and free fatty acids.

The phospholipase A1 enzyme preparation (trade name, Novozym 46016) is produced by submerged fermentation of an *Aspergillus oryzae* production strain carrying a gene encoding phospholipase A1 from *Fusarium venenatum*. The enzyme is subsequently partially purified and concentrated, resulting in a liquid enzyme concentrate, which, in the final preparation, is stabilized, formulated and standardized with glycerol, sucrose, water, sodium benzoate and potassium sorbate. The enzyme activity is measured relative to a porcine pancreas phospholipase A2 (lecitase) standard and is expressed in lecitase units (LEU). Novozym 46016 has a typical activity of 2000 LEU/g, and has the following composition: total organic solids, ~ 2%; water, ~ 51%; glycerol, ~ 35%; sucrose, ~ 10%; ash (mainly sodium chloride), ~ 2%; sodium benzoate, ~ 0.2%; and potassium sorbate, ~ 0.2%.

Novozym 46016 is intended for use in the dairy industry as a processing aid in the manufacture of cheese, to produce modified phospholipids in milk. The modified phospholipids have improved emulsification properties and help to retain more solids in the cheese. The recommended dosage is up to 10 LEU/g milk fat, corresponding to 350 LEU (or 0.175 g Novozym 46016)/l milk if the milk contains 3.5% milk fat.

1.1 Genetic modification

The host strain for the phospholipase A1 gene, the *A. oryzae* BECh2 strain, was derived from *A. oryzae* strain IFO 4177 (synonym A1560). *A. oryzae* is known to contain genes involved in the synthesis of the secondary metabolites cyclopiazonic acid, kojic acid and 3-β-nitropropionic acid, as well as genes involved in the synthesis of aflatoxins. Therefore, in a first step, *A. oryzae* strain A1560 was genetically modified by site-directed disruption of the endogenous amylase and protease genes to allow production of phospholipase A1 without enzymatic side activities. In the following two steps, the modified strain (designated *A. oryzae* JaL 228) was irradiated to remove its potential to produce secondary metabolites. First, the JaL 228 strain was exposed to γ-radiation, resulting in a mutant (designated *A. oryzae* BECh1) that is devoid of genes involved in the synthesis of aflatoxins and cyclopiazonic acid. Subsequently, the BECh1 strain was subjected to ultra-violet radiation, resulting in a mutant (designated *A. oryzae* BECh2) that is impaired in kojic acid synthesis. It is this BECh2 strain that is used as the host strain for the phospholipase A1 gene. When tested under conditions optimal for the production of secondary metabolites, the BECh2 strain did not produce aflatoxins (or the intermediate compounds sterigmatocystin and 5-methoxysterigmatocystin), cyclopiazonic acid or 3-β-nitropropionic acid, and, although it produced kojic acid, it did so only at a level of about 15% of that produced by the A1560 and BECh1 strains.

The phospholipase A1 gene originates from *F. venenatum* CC1-3. Although *F. venenatum* is known to produce secondary metabolites such as trichothecenes, culmorins, enniatins and fusarins, the genetic material transferred to *A. oryzae* is limited to the phospholipase A1 coding sequence. Therefore, the *A. oryzae* production strain cannot produce any secondary metabolites from *F. venenatum.*

The phospholipase A1 gene is cloned into an *A. oryzae* expression plasmid, generating the phospholipase A1 expression plasmid pPFJo142. This expression plasmid is based on the standard *Escherichia coli* vector pUC and contains known and well-characterized DNA sequences. The pPFJo142 expression plasmid was used to transform the *A. oryzae* BECh2 host strain to obtain the *A. oryzae* PFJo142 production strain. The plasmid is stably integrated into *A. oryzae* chromosomal DNA and does not contain antibiotic resistance genes. The inserted DNA also does not encode for or express any substances known to be harmful or toxic. Phospholipase A1 expressed by the production strain has no significant amino acid sequence homology with known allergens or toxins listed in publicly available databases. When analysed for aflatoxin B_1, ochratoxin A, sterigmatocystin, T-2 toxin, zearalenone, cyclopiazonic acid, kojic acid and 3-β-nitropropionic acid, none of these secondary metabolites was detected in two test batches of the enzyme preparation.

1.2 Product characterization

Phospholipase A1 is produced by submerged fed-batch pure culture fermentation of the *A. oryzae* PFJo142 production strain. It is secreted into the

fermentation medium, from which it is recovered and concentrated, and subsequently stabilized, formulated and standardized with glycerol, sucrose, water, sodium benzoate and potassium sorbate. The enzyme preparation is added to milk before the coagulation step in the manufacture of cheese. After coagulation, most of the enzyme is drained off with the whey stream, which is pasteurized, resulting in inactivation of phospholipase A1. Any enzyme remaining in the cheese can no longer function, either because there is no substrate left, or because the substrate is occluded by the solid cheese matrix and therefore unavailable to the enzyme. Cheese can contain the reaction products, lysophospholipids and free fatty acids, which are considered normal constituents of the diet.

The phospholipase A1 enzyme preparation conforms to the *General Specifications and Considerations for Enzyme Preparations Used in Food Processing* prepared by the Committee at its fifty-seventh meeting (Annex 1, reference 156). The enzyme preparation is free from the production organism and recombinant DNA.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

F. venenatum phospholipase A1 was assessed for potential allergenicity by comparing its amino acid sequence with those of known allergens listed in publicly available databases (SWALL and GenBank). No immunologically significant sequence homology was detected. A sequence homology assessment of *F. venenatum* phospholipase A1 with respect to the sequences of known toxins listed in the same databases also revealed no significant homology.

2.2 Toxicological studies

The host organism *A. oryzae* is not pathogenic and has a long history of safe use in food. Enzyme preparations from *A. oryzae* have been evaluated previously by the Committee, which concluded that, since α-amylase and protease from *A. oryzae* are derived from a microorganism that is accepted as a constituent of foods and is normally used in food production, they must be regarded as foods and are thus acceptable for use in food processing (Annex 1, reference 77). An ADI 'not specified' was allocated to lipase from *A. oryzae* (Annex 1, reference 35), as well as to laccase from a recombinant strain of *A. oryzae* (Annex 1, reference 167).

A. oryzae host strains derived from strain A1560 have been used in the construction of several other Novozym enzyme products, including four enzymes derived from the same host strain, *A. oryzae* BECh2, as the phospholipase A1 enzyme. These four enzymes include a modified lipase, glucose oxidase and two xylanases. The DNA introduced into the production strains of these four enzymes is essentially the same as that introduced into the phospholipase A1 production strain *A. oryzae* PFJo142, except for the sequence encoding the specific enzyme. All the Novozym enzyme products, including the four *A. oryzae* BECh2-derived enzymes, were stated to have been assessed for safety (in at least a 13-week study of toxicity in rats treated orally, an assay for mutagenicity in bacteria in vitro and a cytogenetic assay in human lymphocytes in vitro) and approved in many countries (e.g. Australia,

Canada, Denmark and France) or were the subjects of GRAS notices submitted to the United States Food and Drug Administration.

The sponsor concluded that the BECh2 host strain and the production strains derived therefrom constitute a safe strain lineage and that the phospholipase A1 enzyme preparation needed only limited toxicological testing. Accordingly, only two toxicological studies in vitro were performed (see below) with a phospholipase A1 liquid enzyme concentrate (batch PPW 22837; dry matter content, 13.8% w/w), which had not undergone stabilization, formulation or standardization. Additionally, summaries were provided of toxicological studies performed with the other four *A. oryzae* BECh2-derived enzymes. On request, full toxicological data were also provided for one of these four enzymes, a xylanase (called Shearzyme) that had been tested recently. Except for the sequence coding for xylanase (derived from *A. aculeatus*), the DNA introduced into the xylanase production strain is the same as that introduced into the phospholipase A1 production strain. The toxicological studies were performed with one test batch of the xylanase liquid enzyme concentrate (batch PPJ 6867; dry matter content, 12.4% w/w; enzyme activity, 2000 fungal xylanase units/g, total organic solid content, 10.1%; specific gravity, 1.054 g/ml), without formulation or standardization.

2.2.1 Genotoxicity

(a) Phospholipase A1

Phospholipase A1 (batch PPW 22837) was tested for its capacity to induce reverse mutation in vitro in a study that followed OECD test guideline 471 (1997), without a repeat experiment, and was certified for compliance with GLP. Concentrations of 156–5000 mg/ml were tested in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA with and without a 9000 x *g* supernatant from rat liver (S9), by the 'treat-and-plate' method (to avoid problems due to the presence of free amino acids like histidine and tryptophan in the phospoholipase preparation) and by the plate incorporation method for the *E. coli* strain. Cell viability was reduced in the 'treat-and-plate' assay (most pronounced with S9), with concurrent weak reductions in the number of revertants. Growth inhibition in the plate incorporation assay was weak and insignificant at higher concentrations (Pedersen, 2004). The result is impossible to interpret because there was considerable cytotoxicity. Although no explanation was provided for this observation, it might have been due to enzymatic activity on the cells.

(b) Xylanase

The results of three studies of genotoxicity with xylanase (batch PPJ 6867) in vitro are summarized in Table 1. The first study followed OECD test guideline 471 (1997) and the second OECD test guideline 473 (1997). The second and third study were certified for compliance with GLP and QA.

2.2.2 Special studies: Cytotoxicity

The cytotoxic potential of phospholipase A1 (liquid enzyme concentrate, batch PPW 22837) was examined in the neutral red uptake assay in cultured L929 mouse fibroblast cells. This assay for cell survival and viability is based on the ability of

Table 1. Genotoxicity of xylanase in vitro

End-point	Test system	Concentration	Results	Reference
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	156–5000 mg/plate, ±S9	Negative[a]	Pedersen (2003)
Chromosomal aberration	Human lymphocytes	1st experiment: 3200, 4000 or 5000 µg/ml, ±S9 2nd experiment: 2813, 3750 or 5000 µg/ml, ±S9	Negative[b]	Whitwell (2003a)
Micronucleus induction	Human lymphocytes	1st experiment: 3200, 4000 or 5000 µg/ml, ±S9 2nd experiment: 2813, 3750 or 5000 µg/ml, –S9	Negative[c]	Whitwell (2003b)

S9, 9000 x *g* supernatant from rat liver

[a] With and without S9, by the plate incorporation method and, for *S. typhimurium* strain TA1535, also the 'treat-and-plate' method (to avoid problems if the test substance contained significant levels of bioavailable histidine); no cytotoxicity observed

[b] With and without S9. In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. No effects on mitotic index were observed. In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested. With S9, the cells were treated for 3 h and harvested 17 h later. No effects on the mitotic index were observed.

[c] With and without S9. In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 21 h later. In the second experiment, the cells were exposed continuously for 48 h without S9 and then harvested. No micronuclei were induced.

viable cells to incorporate and bind neutral red, a weakly cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Cytotoxicity is expressed as the concentration of test material required to reduce the uptake of neutral red to 50% that of untreated cells after 24 h of exposure (NRU_{50}). The study was certified for compliance with GLP. Phospholipase A1 was not cytotoxic in this assay, given the cell viability of 91–97% at concentrations of 0.3–30 mg/ml phospholipase A1 (in Earle minimum essential medium with 10% fetal bovine serum) as compared with 100% for untreated control cells. Hence, the NRU_{50} value for phospholipase A1 was > 30 mg/ml (Elvig-Jørgensen, 2003).

3. DIETARY EXPOSURE

The phospholipase A1 enzyme preparation is intended for use in the dairy industry to modify milk before coagulation. Experience with this enzyme is currently limited to production of mozzarella and Cheddar cheese, but it could be used for the production of other types of cheese. After coagulation of the cheese, most of the

enzyme is drained off with the whey stream, which is then pasteurized, causing inactivation of the enzyme activity. Whey is a by-product that is usually introduced into the food chain as an ingredient of other processed foods. It can be assumed that the enzyme is distributed evenly to the overall mass, i.e. if 100 l milk typically gives 10 kg of cheese, an estimated 10% of the enzyme would remain in the cheese and 90% would be present in the whey. The portion that may remain in the cheese is inactive due to lack of substrate: within 20 min, more than 95% of the target phospholipids are hydrolysed, and there is no further reaction after 2.5 h.

The possible dietary intake of the enzyme from whey derivatives used as ingredients in processed foods is difficult to assess owing to the large variety of potential uses. Overall dietary intake of the enzyme was assessed only from cheese consumption but on the assumption that all the enzyme added to milk would remain in the cheese. It was also assumed that:

- All cheese is produced with the phospholipase A1 enzyme preparation as a processing aid.
- The enzyme preparation contains 2% total organic solids.
- The dose of phospholipase A1 enzyme preparation was the recommended level, 17.5 g/100 l milk (equivalent to 350 LEU/l milk if the milk contains 3.5% milk fat).
- 100 l of milk typically makes 10 kg of cheese.

Cheese would then contain 0.035 mg of total organic solids per g: (17.5 g x 0.02)/ 10 000.

According to the budget method, the upper physiological consumption of food is 50 g/kg bw per day (Hansen, 1979). A conservative scenario would be consumption of 6.25 g/kg bw per day of cheese (375 g of cheese per day by a person weighing 60 kg). The hypothetical dietary intake of total organic solids from cheese would then be 0.22 mg/kg bw, corresponding to 13 mg for a person weighing 60 kg.

4. COMMENTS

Toxicological data

Only two toxicological studies were performed in vitro with the phospholipase A1 enzyme under evaluation, because the *A. oryzae* BECh2 host strain and the production strain *A. oryzae* PFJo142 derived therefrom were considered to constitute a safe strain lineage. Additionally, summaries were provided of toxicological studies performed with four enzymes derived from the same host strain, *A. oryzae* BECh2: a modified lipase, glucose oxidase and two xylanases. The DNA introduced into the production strains of these enzymes is essentially the same as that introduced into the phospholipase A1 production strain, except for the sequence encoding the specific enzyme. At the request of the Committee, full toxicological data were also provided on one of the four enzymes, a xylanase on which studies had recently been conducted. The studies were a 13-week study of oral toxicity in rats, an assay for mutagenicity in bacteria in vitro and two assays of cytogeneticity in human lymphocytes in vitro.

In the two toxicological studies with the phospholipase A1 enzyme, a test batch of the liquid enzyme concentrate was used, without stabilization, formulation

or standardization. The liquid enzyme concentrate was not cytotoxic in an assay in mammalian cells in vitro. Considerable cytotoxicity was, however, observed in bacteria in vitro, making it impossible to interpret the result. The experiment was not repeated. Although no explanation was given for the observed cytotoxicity, the Committee considered that it might have been the result of enzymatic activity on the cells. In contrast to the finding for phospholipase A1, no cytotoxicity was observed when the enzyme xylanase, which is also derived from the host strain *A. oryzae* BECh2, was tested in the same assay for mutagenicity in bacteria in vitro.

The Committee noted that the materials added to the phospholipase A1 liquid enzyme concentrate for stabilization, formulation and standardization have either been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

Assessment of dietary exposure

When phospholipase A1 is used as a processing aid in the production of cheese, most of the enzyme is drained off with the whey, and only a small amount remains in cheese. Although whey derivatives are known to be used as ingredients in processed foods, it is difficult to assess potential dietary exposure because of the wide variety of uses. On the basis of a conservative estimate of daily consumption of 375 g of cheese by a 60-kg adult and on the assumption that the enzyme is used at the recommended dosage and all total organic solids originating from enzyme preparation remain in the cheese, the dietary exposure would be to 0.22 mg of total organic solids per kg bw per day.

5. EVALUATION

The Committee concluded that the information provided on the enzyme phospholipase A1 was too limited to allow an assessment of its safety. Only two test batches of the enzyme preparation were analysed for secondary metabolites, and, of the two toxicological studies provided, one, the assay for mutagenicity in bacteria in vitro, could not be interpreted owing to considerable cytotoxicity. Given that cytotoxicity was not observed when xylanase, an enzyme derived from the same host strain *A. oryzae* BECh2, was tested in the same assay, the Committee decided not to use the toxicological data provided on xylanase to assess the safety of phospholipase A1. The Committee concluded that, in order to make a proper safety assessment, the results of two adequate studies of genotoxicity (including a test for chromosomal aberration in mammalian cells in vitro) and a study of toxicity in vivo would be needed. Alternatives to toxicity testing in vivo would be the demonstration that no unintended compounds are present in the enzyme preparation or better molecular characterization of the genetically modified microorganism.

6. REFERENCES

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