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PHOSPHOLIPASE A1 FROM *FUSARIUM VENENATUM* EXPRESSED IN *ASPERGILLUS ORYZAE*
CHEMICAL AND TECHNICAL ASSESSMENT (CTA)

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1 Summary

This Chemical and Technical Assessment summarizes the information about the phospholipase A1 enzyme preparation submitted to JECFA by Novozymes A/S in a dossier dated December 10, 2004 (Novozymes, 2004). The document also discusses published information relevant to the production organism, *Aspergillus oryzae*.

Phosholipase A1, described in the Novozymes dossier, catalyses the hydrolysis of the sn-1 ester bond in diacylphospholipids to form 2-acyl-1-lysophospholipids and free fatty acids. The enzyme is manufactured by pure culture fermentation of a genetically modified strain of *A. oryzae* that contains the gene encoding phospholipase A1 derived from *Fusarium venenatum*. Both *A. oryzae* and *F. venenatum* are well-characterized filamentous fungi. The *A. oryzae* production strain was developed from a nonpathogenic *A. oryzae* strain IFO 4177, also known as strain A1560, using recombinant DNA techniques and traditional mutagenesis.

Strain A1560 is capable of producing low levels of toxic secondary metabolites, cyclopiazonic acid, kojic acid, and 3-β-nitropropionic acid, when cultivated in media conducive to the synthesis of these compounds. Strain A1560 is also known to contain non-functional genes related to aflatoxin synthesis. Nevertheless, strain A1560 and its derivatives have been used in the production of enzymes that were shown to be safe for use in food.

The phospholipase A1 production strain was genetically improved by deleting genes related to the synthesis of aflatoxins and cyclopiazonic acid. The strain was also modified to reduce its ability to produce kojic acid. As a result of these genetic modifications, the toxigenic potential of the phospholipase A1 production strain has been substantially reduced in comparison with the A1560 strain. The DNA introduced into the production strain is well-characterized and does not result in the production of harmful substances.

During fermentation, phospholipase A1 is secreted to the fermentation broth. The enzyme is subsequently purified, concentrated and formulated with appropriate food-grade substances. The formulated phospholipase A1, referred to below as the phospholipase A1 enzyme preparation, is marketed under a trade name NOVOZYM® 46016 for use by the dairy industry as a processing aid in the manufacture of cheese. The enzyme preparation is added to cheese milk prior to coagulation in order to modify milk phospholipids. The modified phospholipids have improved emulsifying properties and help to retain more milk solids in the cheese thereby improving the production efficiency. The phospholipase A1 enzyme preparation does not contain measurable levels of secondary metabolites and complies with specifications set out by JECFA (2001) and Food Chemicals Codex (FCC, 2004).

2. Description

Brown liquid.

3 Method of Manufacture

3.1 *Aspergillus oryzae*

A. oryzae is a filamentous fungus that occurs in soil mainly in Japan and China. It has been used in Asia in the production of fermented foods such as soy sauce, miso and the rice wine sake for over 2000 years. At the beginning of the 20th century, *A. oryzae* was used as a source of α-amylase, the first enzyme produced on the industrial scale for use in food (Nielsen *et al*., 1994). Since then, *A. oryzae* has been used in the production of numerous native and heterologous enzymes for use in food processing. *A. oryzae* is not known to be pathogenic and is considered to be a safe source of food-processing enzymes. Some strains of *A. oryzae* may produce low levels of secondary metabolites with low to moderate toxicity 3-β-nitropropionic acid, kojic acid, and cyclopiazonic acid (Barbesgaard et al., 1992; Blumenthal, 2004). The synthesis of these secondary metabolites can be limited or avoided by using appropriate fermentation conditions. According to several recent reports, certain strains of *A. oryzae* contain structural and regulatory genes involved in the synthesis of aflatoxins. Nevertheless, these strains do not produce aflatoxins even under conditions that favor aflatoxin synthesis (Watson, et al., 1999; van der Broek et al., 2001).

3.2 Development of *A. oryzae* host and production strains

The *A. oryzae* production strain, PFJo142, carries the phospholipase A1 gene derived from *Fusarium venenatum* strain CC1-3. *F. venenatum* is capable of producing toxic secondary metabolites such as trichothecenes, fusarins, culmorins, and enniatins under certain fermentation conditions. However, the *F. venenatum* DNA transferred to *A. oryzae* is limited to the phospholipase A1 coding sequence. Therefore, it is not possible for the *A. oryzae* production strain to produce any secondary metabolites from *F. venenatum* and the safety of the *F. venenatum* strain CC1-3 is not relevant to the safety of the phospholipase A1 enzyme preparation.

The *A. oryzae* production strain was developed by transformation of the *A. oryzae* host strain BECh2 with the expression vector pPFJo142. The BECh2 strain is a derivative of the well-known industrial production strain IFO 4177 (also known as strain A1560) originally obtained from the Institute for Fermentation in Osaka, Japan. Strain A1560 was genetically modified by site-directed disruption of three endogenous TAKA amylase genes, one alkaline protease gene, and one neutral metalloprotease I gene. The modified strain was designated as JaL 228. Due to inactivation of the amylase and protease genes, enzymes derived from strain JaL 228 or its derivatives are not expected to contain significant levels of side enzymatic activities.

Strain A1560 contains genes involved in the synthesis of secondary metabolites with low to moderate toxicity, 3-β-nitropropionic acid, kojic acid, and cyclopiazonic acid. These compounds are synthesized under nutritionally limiting conditions rather than under conditions that are optimized for industrial production of enzymes. Strain A1560 also contains a cluster of nonfunctional genes related to the synthesis of aflatoxins. Despite its toxigenic potential, strain A1560 and its derivatives have a long history of use as sources of food-processing enzymes that have been shown to be free of harmful levels of toxic substances.

To reduce the potential for producing secondary metabolites, the *A. oryzae* strain JaL 228 (derived from strain A1560 as described above) was exposed to γ-radiation and screened for cyclopiazonic acid production. A mutant deficient in cyclopiazonic acid synthesis (designated as BECh1) was selected and analysed using standard genetic methods. The genetic analysis showed that the segment of one chromosome containing genes involved in the synthesis of aflatoxins and cylopiazonic acid was deleted. The deletion of these genes is permanent and reversion to the aflatoxin or cyclopiazonic production capability is not possible. Therefore, the BECh1 strain and any strains derived thereof will be incapable of producing aflatoxins and cyclopiazonic acid regardless of the cultivation conditions. To confirm the genetic analysis, the BECh1 strain was assessed under conditions optimized for production of secondary metabolites. Under these conditions, the strain did not produce aflatoxins (or intermediate compounds sterigmatocystin and 5-methoxy-sterigmatocystin), cyclopiazonic acid, or 3-β-nitropropionic acid. Under

the same conditions, the A1560 strain did not produce aflatoxins and related compounds (as expected) but produced cyclopiazonic and kojic acids, and very low levels of 3-β-nitropropionic acid.

The BECh1 strain was subsequently subjected to UV mutagenesis. A mutant impaired in kojic acid synthesis was selected, designated as BECh2, and used as a host strain for the phospholipase A1 gene. The BECh2 strain was tested for production of secondary metabolites under optimized conditions. The strain produced only kojic acid at a level of approximately 15% of that produced by the A1560 and BECh1 strains cultivated under the same conditions. The phospholipase A1 production strain, which was developed from the BECh2 host strain, is expected to retain these characteristics. To confirm that no secondary metabolites of toxicological concern were formed during phospholipase A1 production, two batches of the enzyme were analyzed for aflatoxin B_1, ochratoxin A, sterigmatocystin, T-2 toxin, zearalenon, cyclopiazonic acid, kojic acid, and 3-β-nitropropionic acid. None of these secondary metabolites was detected in the phospholipase A1 enzyme preparation.

The BECh2 strain was used by Novozymes as a host strain in the construction of production strains for xylanases from *Aspergillus aculeatus* and *Thermomyces lanuginosus,* glucose oxidase from *A. niger*, and triacylglycerol lipase from *T. lanuginosus/Fusarium oxysporum*. The DNA introduced into these production strains was essentially the same as that introduced into the phospholipase A1 production strain, PFJo142, except for the DNA sequences encoding the individual enzymes.

3.3 Expression vector

The expression vector pPFJo142 used for transformation of the *A. oryzae* host strain BECh2 contains the replication origin derived from the standard *Escherichia coli* vector pUC19. The vector also contains the *URA3* gene from *Saccharomyces cerevisiae* (baker's yeast) that compensates the *pyrF* mutation in the *E. coli* strain used as an intermediate host for the construction of the expression vector. The *pyrF* mutant requires uridine for growth. This requirement is alleviated in the *E. coli* transformants carrying the *URA3* gene provided on the expression vector. The *URA3*-based selection system eliminated the need for use of an antibiotic resistance selectable marker.

The expression vector carries the phospholipase A1 coding sequence from *F. venenatum* combined with regulatory sequences, promoter and terminator. The phospholipase A1 promoter consists of two modified promoter sequences, Pna2 and Pna2/TPI. The Pna2 sequence is the neutral amylase II promoter from *A. niger* from which the TATA box was deleted. The Pna2/TPI sequence consists of the Pna2 promoter in which the 5' nontranslated region was replaced with the corresponding region from the *A. nidulans* triose phosphate isomerase promoter. The transcription terminator is the amyloglucosidase gene terminator from *A. niger.*

The expression vector also carries the *amdS* gene from *Aspergillus nidulans* which serves as a selectable marker in *A. oryzae*. It encodes acetamidase, an enzyme that catalyses the hydrolysis of acetamide to acetic acid and ammonia which are subsequently used by *A. oryzae* as carbon and nitrogen sources. The *A. oryzae* cells transformed with the expression vector are able to grow on acetamide as a sole nitrogen source.

3.4 Transformation and selection

The phospholipase A1 expression vector, pPFJo142, was introduced into the host strain BECh2 by incubating the vector with the host protoplasts. The transformants were selected on acetamide and screened for phospholipase activity. A transformant designated as PFJo142 was selected as a phospholipase A1 production strain.

3.5 Characterization of the production strain

The expression plasmid is stably integrated into *A. oryzae* chromosomal DNA and, as such, is poorly mobilizable for transfer to other organisms. The genetic stability of the production strain PFJo142 during large-scale fermentation was confirmed using Southern blot analysis.

The DNA introduced into the *A. oryzae* host strain is well characterized. It does not contain antibiotic resistance genes or any DNA sequences that would result in the production of toxic substances. The production strain PFJo142 was developed from the host strain BECh2 that was genetically modified by deleting genes involved in the synthesis of aflatoxins and cyclopiazonic acid and by selecting a mutant with substantially reduced capability of producing kojic acid. The safety of the production strain PFJo142 as a source of phospholipase A1 was confirmed by analyzing two batches of the phospholipase A1 preparation for aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin, zearalenon, cyclopiazonic acid, kojic acid, and 3-β-nitropropionic acid. Of these mycotoxins, only low levels of kojic acid and 3-β-nitropropionic acid could theoretically be produced. Nevertheless, neither kojic acid and 3-β-nitropropionic acid nor the remaining mycotoxins were detected. These results confirm that the toxigenic potential of the phospholipase A1 production strain has been minimized through genetic modifications. Although the strain still contains genes involved in the synthesis of kojic acid and 3-β-nitropropionic acid, the production of these secondary metabolites is impaired even under conditions optimized for the synthesis of secondary metabolites and, based on the analytical data for the phospholipase A1 preparation, it does not occur under the enzyme manufacturing conditions.

3.6 Fermentation, recovery, and formulation

Phospholipase A1 is produced by submerged fermentation of the production strain PFJo142 using a fermentation medium composed of food grade materials providing adequate supply of carbon, nitrogen, and micronutrients necessary for growth. The fermentation is conducted under controlled conditions and monitored for microbial contamination. If contamination is detected, the fermentation is terminated. Phospholipase A1 is secreted to the fermentation broth. After the fermentation has been completed, the cell mass is separated from the broth using drum filtration or centrifugation. The clear broth is then subjected to ultrafiltration and/or evaporation to concentrate and purify the enzyme. During ultrafiltration, low molecular weight components of the broth are partially removed. To remove the residual microorganisms and insoluble components of the fermentation broth, the enzyme concentrate is subjected to pre-germ and germ filtration. The filtrate is mixed with glycerol and sucrose to prevent microbial degradation. The stabilized concentrate is then blended with water, sodium benzoate and potassium benzoate and standardized according to the product specifications.

4 Characterization

4.1 Phospholipase A1

The phospholipase A1 described in the Novozymes' dossier catalyses the hydrolysis of sn-1 ester bonds in diacylphospholipids with the formation of 2-acyl-1-lysophospholipids and free fatty acids. Phospholipase A1 is described by the International Union of Biochemistry and Molecular Biology (IUBMB, Web Version) as follows:

Common name: phospholipase A1
Reaction: phosphatidylcholine + H_2O = 2-acylglycerophosphocholine + a carboxylate
Systematic name: phosphatidylcholine 1-acylhydrolase
EC: 3.1.1.32

The Chemical Abstract Service Registry Number (CAS No.) of phospholipase A1 is 9043-29-2. The phospholipase A1 activity is measured relatively to a porcine pancreas phospholipase standard using lecithin as a substrate. Phospholipase A1 catalyses the hydrolysis of lecithin to lyso-lecithin and a free fatty acid. The liberated fatty acid is titrated with 0.1N sodium hydroxide under standard conditions

(pH=8.00; 40.0^{0}C±0.5). The activity of phospholipase A1 is determined as a rate of sodium hydroxide consumption during neutralization of the fatty acid and is expressed in Lecitase activity units (LEU). One LEU is defined as the amount of enzyme that under standard conditions (pH=8.00; 40.0^{0}C ±0.5) gives the same rate of sodium hydroxide consumption (µmol/min) as a Lecitase (phospholipase) standard diluted to a nominal activity of 1 LEU/g. The quantification limit of the method is approximately 1.5 LEU/ml.

The amino acid sequence of phospholipase A1 was compared to the amino acid sequences of known allergens contained in the SWALL (SWISSPROT and TrEMBL) and GeneBank databases. According to Metcalf et al. (1996), an immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids. No immunologically relevant sequence identity between phospholipase A1 and known allergens was detected. Therefore, the probability that phospholipase A1 is an allergen is very low. The amino acid sequence of phospholipase A1 was also compared to the amino acid sequences of known protein toxins. No significant sequence homology between phospholipase A1 and protein toxins was found.

4.2 Phospholipase A1 enzyme preparation

The phospholipase A1 enzyme preparation is formulated for use in dairy applications. It does not contain significant levels of side activities because the endogenous amylase and protease genes were deleted during construction of the phospholipase A1 production strain. The phospholipase A1 preparation is marketed under a trade name NOVOZYM® 46016. The typical activity of the product is 2000 LEU/g. The typical composition of NOVOZYM® 46016 is (approximately):

Total Organic Solids (TOS):	2%
Water	51%
Glycerol	35%
Sucrose	10%
Ash (mainly NaCl)	2%
Sodium benzoate	0.2%
Potassium sorbate	0.2%

TOS is defined as 100% - water - ash - diluents (NRC/NAS, 1981).

The phospholipase A1 enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing prepared at the 57th JECFA and published in FNP 52, Addendum 9 (JECFA, 2001). The product also conforms to the General and Additional Requirements set forth in the Food Chemicals Codex, 5th edition (FCC, 2004).

5 Functional uses

The phospholipase A1 enzyme preparation is used in the dairy industry as a processing aid during cheese manufacturing. It is added to cheese milk before the coagulant and allowed to modify phospholipids. Approximately 95% of the substrate phospholipids are modified after 20 minutes. Modified phospholipids have improved emulsifying properties and help to retain more milk components in the cheese, thereby increasing cheese production efficiency. The phospholipase A1 enzyme preparation is added to cheese milk at minimum levels to achieve the intended technical effect. The recommended use level is up to 10 LEU/g milk fat. For milk that contains 3.5% fat, 10 LEU/g fat corresponds to 17.5 g of the phospholipase A1 enzyme preparation per 100 litres of cheese milk.

6 Reactions and fate in food

After the coagulation of cheese, most of the phospholipase A1 enzyme preparation is drained off with whey. Whey is pasteurized causing inactivation of phospholipase A1. A minor portion of the enzyme may remain in cheese. However, cheese is depleted of the substrate phospholipids and the residual phospholipase A1 can no longer perform its catalytic function. Cheese may contain the reaction products, lysophospholipids and free fatty acids, which are considered normal constituents of the diet.

7 References

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