ASPARAGINASE FROM *ASPERGILLUS NIGER* EXPRESSED IN *A. NIGER*
Chemical and Technical Assessment (CTA)

Prepared by Zofia Olempska-Beer, Ph.D. for the 69th JECFA

1. Summary

This Chemical and Technical Assessment summarizes data and information on the asparaginase enzyme preparation submitted to JECFA by DSM Food Specialties[1] in a dossier dated 10 December 2007 (DSM, 2007). This document also discusses published information relevant to asparaginase and the production organism, *Aspergillus niger*.

Asparaginase is an enzyme that catalyses the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. The asparaginase enzyme described in the DSM's dossier is intended for use as a processing aid to reduce in food the levels of free L-asparagine, which is a major precursor in the formation of the food contaminant acrylamide. Acrylamide is formed from L-asparagine and reducing sugars in carbohydrate-containing foods that are heated above 120°C. Examples of such foods include bread and other baked goods, fried or baked potato products, and reaction flavors. Asparaginase will be added to food prior the heating step. During heating, the enzyme will be denatured and thereby inactivated.

Asparaginase is manufactured by pure culture fermentation of a genetically modified strain of *A. niger* that contains multiple copies of the asparaginase gene derived from *A. niger*. *A. niger* is a filamentous fungus that commonly occurs in the environment and is considered to be nonpathogenic. The asparaginase production strain was constructed by transformation of the *A. niger* host strain DS 51563 with DNA fragments derived from two plasmids, one containing the asparaginase gene from *A. niger* and the other containing the acetamidase gene from *Aspergillus nidulans*. The acetamidase gene was used as a selectable marker to identify transformants and was subsequently removed from the production strain. As a result, the asparaginase production strain contains multiple copies of the *A. niger* asparaginase gene but no other heterologous genes. The strain complies with the criteria suggested by the Organisation for Economic Co-operation and Development (OECD) for r-DNA GILSP (Good Industrial Large Scale Practice) microorganisms and cellular cultures (OECD, 1992).

Asparaginase is secreted to the fermentation broth and is subsequently purified and concentrated. For commercial purposes, the enzyme concentrate is formulated and standardized into either a liquid or a granulated preparation using appropriate food-grade substances. The asparaginase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006).

To assess potential allergenicity, the amino acid sequence of asparaginase was compared to the sequences of known allergens using bioinformatics criteria recommended in the Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). No amino acid sequence homology that would suggest that asparaginase is an allergen was detected.

2. Description

Yellow to brown clear liquid or off-white granulates.

[1] A. Fleminglaan 1, Delft, P.O. Box 1,2600 MA Delft, The Netherlands

3. Method of manufacture

3.1. Aspergillus niger

A. niger is a filamentous fungus that commonly occurs in the environment and is generally regarded as nonpathogenic. It grows aerobically on organic matter and tolerates a wide range of temperature and pH. *A. niger* has a long history of use as a source of citric acid and enzymes used in food processing. In the U.S., citric acid derived from several sources including *A. niger* is regulated as a Generally Recognized as Safe (GRAS) food ingredient (21 CFR 184.1033). Carbohydrase and cellulase from *A. niger* are regulated as secondary direct food additives (21 CFR 173.120) and recombinant calf chymosin produced from *A. niger* var. *awamori* is regulated as a GRAS food ingredient (21 CFR 184.1685). JECFA evaluated the safety of several enzymes derived from *A. niger*. An ADI (acceptable daily intake) "not specified" was established for glucose oxidase and catalase at the 18th meeting (JECFA, 1974), for amyloglucosidase, carbohydrase, hemicellulase, β-glucanase, and pectinase at the 35th meeting (JECFA, 1990), and for recombinant calf chymosin at the 37th meeting (JECFA, 1991).

The safety of *A. niger* has been discussed in several recent review papers (Schuster et al., 2002; van Dijck at al., 2003; Blumenthal, 2004; Olempska-Beer et al., 2006). According to the information summarized in these papers, a relatively small number of known *A. niger* strains (3%-10%) are able to produce a mycotoxin with toxic properties, ochratoxin A, under certain fermentation conditions. Some strains of *A. niger* can also produce other secondary metabolites that are not considered to be mycotoxins such as nigragillin, nigerazine B, malformins (cyclic peptides), naphto-γ-pyrones, and oxalic acid. It has been suggested that *A. niger* strains intended for use in the production of enzymes and other substances used in food be thoroughly characterized and tested for their ability to produce mycotoxins.

3.2. Asparaginase production strain

The asparaginase production strain DS 53180 (also known as ASP-72) was obtained by insertion of multiple copies of the *A. niger* asparaginase gene into a host strain DS 51563 (also known as ISO-528). The *A. niger* strain DS 51563 was derived from strain GAM-53 (DS 03043), which is a descendant of the original *A. niger* strain NRRL 3122 (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization and Research, Peoria, IL, USA; previously National Regional Research Laboratory Culture Collection). The strain is currently deposited in the American Type Culture Collection (Gaithersburg, MD, USA) as strain ATCC-22343.

The GAM-53 strain was selected by DSM in 1982 for its enhanced production of glucoamylase, an enzyme widely used in the starch processing industry. The strain was taxonomically identified as *A. niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures. The GAM-53 strain contains seven glucoamylase (*gla*A) genes. The strain was modified by deleting all seven glucoamylase loci and inserting unique restriction sites, thereby creating "plug sites" for targeted integration of heterologous genes. Other modifications included inactivation of the protease gene (to reduce proteolysis of enzymes secreted to the fermentation broth) and deletion of two amylase genes. Strain DS 51563 is one of the "plug bug" strains derived from the GAM-53 strain for production of heterologous enzymes.

The DS 51563 host strain was co-transformed with DNA expression cassettes derived from the asparaginase expression plasmid (pGBTOPASP-1) and the selectable marker plasmid (pGBAAS-3). The expression plasmid contained the asparaginase expression cassette, which consisted of the asparagine gene (*aspA)* derived from the *A. niger* strain GAM-8, and the glucoamylase promoter and terminator both derived from the parental *A. niger* strain GAM-53. The selectable marker plasmid contained the expression cassette consisting of the *amdS* gene from *Aspergillus nidulans*, the glucoamylase promoter and terminator from the GAM-53 strain, and the promoter of the *A.*

nidulans glyceraldehyde-3-phosphate dehydrogenase (*gdpA*) gene. Both plasmids also contained DNA sequences from *E. coli.* However, these sequences were removed before transformation.

The transformed cells were selected by their ability to grow on acetamide as a sole carbon source due to the presence of acetamidase encoded by the *amdS* gene. The transformants were then plated on fluoro-acetamide (a toxic analog of acetamide) and a surviving transformant was selected from which the *amdS* gene was spontaneously deleted as a result of an internal recombination event. Subsequently, the empty glucoamylase (Δ*glaA*) loci were filled up with the asparaginase expression cassettes by a naturally occurring process of gene conversion. The resulting strains were screened and one transformant containing an adequate copy number of the asparaginase gene was selected for commercial production of asparaginase and designated DS 53180. The strain was confirmed to be *A. niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures. The strain complies with the criteria suggested by the Organisation for Economic Co-operation and Development (OECD) for r-DNA GILSP (Good Industrial Large Scale Practice) microorganisms and cellular cultures (OECD, 1992).

The asparginase production strain was tested for its potential to produce toxic metabolites. In one experiment, the strain was grown under conditions generally known to induce toxins. The cells were harvested and extracted and the extract was analyzed for a broad range of toxins. The results showed that the strain produced only naphtho-γ-pyrones at measurable levels. In another experiment, the production strain was grown under normal production conditions. Both the fermentation broth and supernatant were extracted and analyzed. There was no indication that any *A. niger* secondary metabolites (including ochratoxins) were present in the samples.

3.3. Fermentation, recovery, and formulation

Asparaginase is produced by submerged fermentation of the asparaginase production strain using a fermentation medium composed of food-grade (or equivalent) raw materials. The fermentation is conducted under controlled conditions in accordance with current Good Manufacturing Practice. Asparaginase is secreted to the fermentation broth and is subsequently purified and concentrated. For commercial purposes, the concentrate is formulated into either a liquid or dry enzyme preparation using appropriate food-grade materials.

4. Characterization

4.1. Asparaginase

Asparaginase catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The Chemical Abstract Service Registry Number (CAS No.) of asparaginase is 9015-68-3. Asparaginase is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB, online edition) as follows:

Accepted name:	asparaginase
Other name(s):	asparaginase II; L-asparaginase; colaspase; elspar; leunase; crasnitin; α-asparaginase
Reaction:	L-asparagine + H_2O = L-aspartate + NH_3
Systematic name:	L-asparagine amidohydrolase
EC:	3.5.1.1

Asparaginase activity is determined by measuring the rate of hydrolysis of L-asparagine to L-aspartic acid and ammonia under standard conditions (pH=5.0; 37^{o}C). The liberated ammonia subsequently reacts with phenol nitroprusside and alkaline hypochlorite resulting in a blue color. This is known as Berthelot reaction. The activity of asparaginase is determined by measuring absorbance of the reaction mixture at 600 nm. Asparaginase activity is expressed in ASPU activity

units. One ASPU is defined as the amount of asparaginase that liberates one micromole of ammonia from L-asparagine per minute under standard conditions (pH=5.0; 37°C).

Asparaginase from *A. niger* described in the DSM's dossier consists of 378 amino acids. It is a glycoprotein with an apparent molecular mass of about 50 kDa and iso-electric point of approximately 3.6. Asparginase is active between 20 and 60°C and has a temperature optimum around 50°C at pH 5. The enzyme is quickly inactivated when the temperature is raised to 70°C. At 37°C, asparaginase is optimally active at pH between 4 and 5. Asparaginase activity is relatively stable in the presence of salt (NaCl). Nearly 80% of the activity is still retained at salt concentrations approaching 10% (measured after 30 min at 37°C and pH 5).

Asparaginase from *A. niger* was assessed for potential allergenicity using bioinformatics criteria recommended in the Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). According to the FAO/WHO report, cross-reactivity with known allergens should be considered if the protein has either more than 35% sequence identity in any amino acid window of 80 amino acids, or shows an identity of at least six contiguous amino acids. The amino acid sequence of asparaginase was compared to the amino acid sequences of known allergens in the SDAP database from the University of Texas Medical Branch (http://fermi.utmb.edu/SDAP/sdap_who.html). The database contains all allergens from the International Union of Immunological Societies website (http://www.allergen.org) supplemented with data from the literature and protein databases (SwissProt, PIR, NCBI, and PDB). The results of the sequence comparisons showed no amino acid homology that would suggest cross-reactivity of asparaginase with known allergens.

Enzymes with asparaginase activity occur in microorganisms, animals, and plants. Asparaginase from *Aspergillus oryzae* expressed in a genetically engineered strain of *A. oryzae* was evaluated by JECFA at its 68th meeting (JECFA, 2007).

4.2. Asparaginase enzyme preparation

The asparaginase enzyme preparation is marketed under a trade name PreventAse™. It is formulated either as a liquid preparation or a dry granulated product. The starting material for both forms is the enzyme concentrate after ultra-filtration. The concentrate is subjected to additional purification steps, such as polish and germ filtration. To obtain the liquid preparation, the concentrate is stabilized and standardized by addition of glycerol to a final activity of 2300 to 2800 ASPU/g. To obtain a dry enzyme preparation, the enzyme concentrate is spray-dried and granulated with either maltodextrin or wheat flour and is subsequently standardized to an activity of 2300 to 2800 ASPU/g with the same carrier. The total organic solids (TOS) content of the final product is expected to vary from 6 to 10%. The TOS content is used in calculating exposure to enzyme preparations and is defined as follows:

TOS (%) = 100 – (A + W + D)

where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients (NAS/NRC, 1981; JECFA, 2006). TOS consists of the enzyme of interest and residues of organic materials, such as proteins, peptides, and carbohydrates, derived from the production organism and the manufacturing process.

The asparaginase enzyme preparation conforms to the General Specifications for Enzyme Preparations Used in Food Processing (JECFA, 2006). It contains neither viable cells of the production organism nor significant levels of side activities.

5. Functional uses

Asparaginase is intended for use as a processing aid during food manufacture to reduce the level of L-asparagine by its hydrolysis to L-aspartic acid and ammonia. Free L-asparagine present in food is the main precursor of acrylamide, which is considered to be a probable human carcinogen (JECFA, 2006a). Acrylamide is formed from L-asparagine and reducing sugars primarily in starchy foods that are baked or fried at temperatures above 120^{o}C.

Asparginase will be used during preparation of carbohydrate-rich foods that are major sources of dietary acrylamide, such as bread and other cereal-based products, baked and fried potato-based products, and reaction flavours (also known as "thermal process flavours"). The levels of L-asparagine would be reduced in these foods prior to heating, thereby reducing the availability of L-asparagine for acrylamide formation. Asparaginase will be used in food ingredients at levels shown in Table 1 below. The enzyme will be inactivated by denaturing during the heating step. The estimated TOS residues in the final food (including denatured asparaginase) are provided in Table 1.

Table 1. Asparaginase use levels and TOS residues in the final food

Final food	Use level in food ingredient	TOS residue in the final food
Bread	77-385 ASPU/kg flour	1.48-9.97 mg/kg
Cereal-based products	20-850 ASPU/kg flour	0.14-23.1 mg/kg
Potato flour-based products	500-15000 ASPU/kg flour	2.85-428 mg/kg
Savory ingredients	4700-6200 ASPU/kg reaction flavour	2.68-3.54 mg/kg

DSM conducted laboratory-scale trials to asses the levels of L-asparagine and acrylamide in foods prepared with the addition of asparaginase. Acrylamide was analysed by gas chromatography with mass spectrometric detection while L-asparagine was analysed using an HPLC methodology. The formation of acrylamide was reduced by 36% to 75% in bread crusts, by 86% to 92% in other cereal-based products, such as fritters, donuts, Dutch honey cake, and crackers, and by up to 93% in potato-based products. The effects of asparaginase were assessed in potato-based dough as a function of the incubation time at room temperature. The level of L-asparagine was reduced up to 96% after 70 min of incubation. A similar reduction in the levels of acrylamide measured after frying was observed.

Reaction flavours are prepared by heating ingredients, of which at least one contains amino nitrogen, and another is a reducing sugar. Reducing sugars typically used in the preparation of the reaction flavours include ribose, xylose, arabinose, glucose, fructose, lactose, and sucrose. The sources of amino nitrogen include meat extracts, hydrolysed vegetable proteins, and yeast extracts. All these products contain free L-asparagine. The treatment of yeast extract with asparaginase effectively removed L-asparagine. The levels of acrylamide in a roasted beef reaction flavour prepared with the asparaginase-treated yeast extract were reduced by 73-80% comparing with the untreated control flavour.

6. Reactions and fate in food

Asparaginase will be used during food processing prior to heat treatment. The enzyme would be subsequently inactivated by denaturation during heat processing, such as baking or frying. As a result of the catalytic activity of asparaginase, low levels of L-aspartic acid and ammonia are expected to be formed in food. Both compounds are present in the human diet.

7. References

Blumenthal, C.Z., 2004. Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae,* and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul. Toxicol. Pharmacol. 39, 214-228.

DSM, 2007. DSM Food Specialties. Asparaginase from *Aspergillus niger* expressed in *Aspergillus niger*. 10 December 2007. A dossier submitted to JECFA for consideration at the 69th meeting, Rome, Italy, 17-26 June 2008.

FAO/WHO, 2001. Evaluation of Allergenicity of Genetically Modified Foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22-25 January 2001, Food and Agricultural Organization of the United Nations (FAO), Rome, Italy. (http://www.who.int/foodsafety/publications/biotech/ec_jan2001/en/print.html)

IUBMB, online edition. International Union of Biochemistry and Molecular Biology. (http://www.chem.qmul.ac.uk/iubmb/enzyme/search.html)

JECFA, 1974. Evaluation of certain food additives and contaminants. (Eighteen Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 557. (http://www.who.int/ipcs/publications/jecfa/reports/en/index.html)

JECFA, 1990. Evaluation of certain food additives and contaminants. (Thirty-fifth Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789. (http://www.who.int/ipcs/publications/jecfa/reports/en/index.html)

JECFA, 1991. Evaluation of certain food additives and contaminants. (Thirty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806. (http://www.who.int/ipcs/publications/jecfa/reports/en/index.html)

JECFA, 2006. Joint FAO/WHO Expert Committee on Food Additives. "General Specifications and Considerations for Enzyme Preparations Used in Food Processing." Prepared at the 67th meeting (Rome, Italy, 20-29 June 2006). FAO JECFA Monographs 1, Vol. 4. Available at: http://www.fao.org/ag/agn/jecfa-additives/docs/enzymes_en.htm

JECFA, 2006a. Evaluation of certain food additives and contaminants. (Sixty-fourth Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 930. (http://www.who.int/ipcs/publications/jecfa/reports/en/index.html)

JECFA, 2007. Evaluation of certain food additives and contaminants. (Sixty-eight Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 947. (http://www.who.int/ipcs/publications/jecfa/reports/en/index.html)

NRC/NAS, 1981. The 1978 Enzyme Survey Summarized Data. National Research Council/National Academy of Sciences. Washington, D.C., USA. U.S. Department of Commerce. National Technical Information Service.

OECD, 1992. Safety Considerations for Biotechnology 1992. Organisation for Economic Co-operation and Development. Part One. Elaboration of Criteria and Principles for Good Industrial Large-Scale Practice (GILSP). (http://www.oecd.org/dataoecd/8/3/2375496.pdf)

Olempska-Beer, Z.S., Merker, R.I., Ditto, M.D., DiNovi, M.J., 2006. Food-processing enzymes from recombinant microorganisms – a review. Regul. Toxicol. Pharmacol. 45, 144-158.

Shuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P.W.M., 2002. On the safety of *Aspergillus niger* - a review. Appl. Microbiol. Biotechnol. 59, 426-435.

Van Dijck, P.W.M., Selten, G.C.M., Hempenius, R.A., 2003. On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains. Regul. Toxicol. Pharmacol. 38, 27-35.