

**ASPARAGINASE FROM *ASPERGILLUS ORYZAE* ENCODED BY THE
ASPARAGINASE GENE FROM *A. ORYZAE*
Chemical and Technical Assessment**

Prepared by Zofia Olempska-Beer, Ph.D.

I. Summary

This Chemical and Technical Assessment summarizes data and information on the asparaginase enzyme preparation submitted to JECFA by Novozymes A/S in a dossier dated November 9, 2006 (Novozymes, 2006). This document also discusses published information relevant to the production organism, *Aspergillus oryzae*.

Asparaginase catalyses the hydrolysis of the amino acid asparagine to aspartic acid and ammonia. The asparaginase enzyme preparation described in the Novozymes' dossier is intended for use during food processing to reduce the level of asparagine and thereby to decrease the risk of acrylamide formation. Acrylamide is formed in food from asparagine and reducing sugars when food products are baked or fried at temperatures above 120°C.

Asparaginase is manufactured by pure culture fermentation of a genetically modified strain of *A. oryzae*, which expresses the asparaginase gene derived from *A. oryzae*. The *A. oryzae* production strain was derived 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 enzyme preparations that were shown to be safe for use in food.

Strain A1560 was subjected to numerous genetic modifications using both classical mutagenesis and molecular biology to reduce its capability of producing secondary metabolites as well as extracellular enzymes. As a result of these modifications, a host strain, designated as BECh2, was developed. The BECh2 strain is devoid of genes involved in the synthesis of aflatoxins and cyclopiazonic acid due to the deletion of a large DNA segment carrying these genes. The strain is also impaired in its capability to produce kojic acid and 3-β-nitropropionic acid. When cultivated in media designed to induce the synthesis of mycotoxins, strain BECh2 produced kojic acid at a level of approximately 15% of that produced by strain A1560 and essentially no 3-β-nitropropionic acid. The BECh2 strain was also modified by inactivation of three endogenous amylase and two protease genes. The inactivation of these genes prevents the synthesis of extracellular amylases and proteases that would be secreted to the fermentation medium during production of asparaginase.

The BECh2 strain was subsequently used as a host for the asparaginase gene. The asparaginase gene was isolated from strain A1560 and introduced into the BECh2 strain on the expression vector, which was integrated into genomic DNA of the host strain. All DNA comprising the expression vector is well-characterized and does not result in the production of harmful substances. The BECh2 strain was also used as a host to construct a number of enzyme production strains, including one expressing the phospholipase A1 gene from *Fusarium*

venenatum. The specifications for phospholipase A1 from *Fusarium venenatum* expressed in *Aspergillus oryzae* were established by JECFA at the 65th meeting (JECFA, 2005).

Asparaginase is produced during fermentation of the asparaginase production strain and is secreted to the fermentation broth. The enzyme is subsequently purified, concentrated, and formulated with appropriate food-grade substances. To confirm that no kojic and 3- β -nitropropionic acids are formed under the fermentation conditions used for asparaginase production, the asparaginase preparation was tested for the presence of these secondary metabolites. Neither kojic acid nor 3- β -nitropropionic acid were detected in the enzyme preparation.

The formulated asparaginase is marketed under the name Acrylaway[®] L for use as a processing aid during food manufacture to convert asparagine to aspartic acid and reduce the formation of acrylamide. Asparaginase will be primarily used during production of foods processed at high temperatures, such as potato chips, French fries, and dough-based products, for example, bread, cookies, crackers, or pretzels. The asparaginase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006).

2. Description

Light 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. 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 (reviewed by Barbesgaard *et al.*, 1992; Blumenthal, 2004; Olempska-Beer *et al.*, 2006). 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 did not produce aflatoxins even under conditions that favor aflatoxin synthesis (Watson, *et al.*, 1999; van der Broek *et al.*, 2001; Tominaga *et al.*, 2006).

3.2. *Asparaginase production strain*

The asparaginase production strain was derived from the well-known *A. oryzae* industrial strain IFO 4177 (also known as A1560) originally obtained from the Institute for Fermentation in Osaka, Japan. Strain A1560 is known to contain genes involved in the synthesis of 3- β -nitropropionic acid, kojic acid, and cyclopiazonic acid. These secondary metabolites are synthesized under nutritionally limiting conditions rather than under conditions that are optimized

for industrial production of enzymes. Strain A1560 also contains inactive genes related to aflatoxin synthesis.

Strain A1560 was genetically modified by site-directed disruption of three endogenous amylase genes, one alkaline protease gene, and one neutral metalloprotease I gene. The inactivation of these genes prevents the synthesis of extracellular amylases and proteases that would be secreted to the fermentation medium during production of asparaginase. The modified strain was designated as JaL 228.

To reduce the potential for producing secondary metabolites, strain JaL 228 was exposed to γ -radiation and screened for cyclopiazonic acid production. A mutant deficient in cyclopiazonic acid synthesis (designated as BECh1) was selected and analyzed using standard genetic methods. The genetic analysis showed that the segment of one chromosome containing genes involved in the synthesis of aflatoxins and cyclopiazonic acid was deleted. The deletion of these genes is permanent and reversion to the aflatoxin or cyclopiazonic acid 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 results of the genetic analysis, the BECh1 and A1560 strains were assessed under conditions optimized for production of secondary metabolites. Under these conditions, neither strain produced aflatoxins and related compounds (sterigmatocystin and 5-methoxy-sterigmatocystin), as expected. While the A1560 strain produced cyclopiazonic acid and kojic acid, the BECh1 strain produced only kojic acid. Additional growth media were subsequently developed to test the BECh1 strain for production of 3- β -nitropropionic acid. The compound was produced at a trace level only under one set of very specific cultivation conditions.

The BECh1 strain was subsequently subjected to UV mutagenesis. A mutant impaired in kojic acid synthesis was selected and designated as BECh2. The BECh2 strain was tested (along with the BECh1 and A 1560 strains) 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 BECh2 strain was also repeatedly tested for production of 3- β -nitropropionic acid in several mycotoxin-inducing media in combination with various cultivation conditions such as temperature and aeration. The compound was detected only under one combination of the cultivation conditions.

The BECh2 strain was used as a host for the asparaginase gene, which was derived from the *A. oryzae* strain A1560. The asparaginase production strain was obtained by transformation of the BECh2 strain with the expression plasmid pCaHj621. The expression plasmid was stably integrated into genomic DNA of the host strain. All DNA sequences comprising the expression plasmid are known. The plasmid carries the origin of replication from the *Escherichia coli* vector pUC19 and the asparaginase coding sequence from *A. oryzae* strain A1560 placed under the control of regulatory sequences, promoter and terminator. The promoter and terminator sequences were derived from *Aspergillus niger* and *Aspergillus nidulans*. Both the promoter and terminator sequences are well-characterized and do not contain any coding DNA. The expression vector also contains the *URA3* gene from baker's yeast *Saccharomyces cerevisiae* and the *amdS* gene from *A. nidulans*. Both genes are well-characterized and serve as selectable markers during the construction of the asparaginase production strain. The expression plasmid does not contain antibiotic resistance genes or any other coding sequences besides those discussed above.

The asparaginase production strain, designated as pCaHj621/BECh2#10, is expected to retain the secondary metabolite profile of the BECh2 host strain, i.e., to be capable of producing reduced

levels of kojic acid and trace levels of 3- β -nitropropionic acid under specific cultivation conditions. However, the synthesis of these secondary metabolites is not expected under conditions normally used in the large-scale production of enzymes intended for use in food. To confirm that no kojic and 3- β -nitropropionic acids were formed during asparaginase production at toxicologically significant levels, one batch of the asparaginase enzyme preparation was analyzed for these compounds. Neither kojic acid nor 3- β -nitropropionic acid were detected at the respective detection limits of 1.4 mg/kg and 0.6 mg/kg.

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*, triacylglycerol lipase from *T. lanuginosus/Fusarium oxysporum*, and phospholipase A1 from *Fusarium venenatum*. The DNA introduced into these production strains was essentially the same as that introduced into the asparaginase production strain, except for the DNA sequences encoding the individual enzymes.

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 materials. The enzyme is secreted to the fermentation broth. The fermentation is conducted under controlled conditions and monitored for microbial contamination. If contamination is detected, the fermentation is terminated. 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 enzyme is then formulated with glycerol, sodium benzoate and potassium sorbate.

4. Characterization

4.1. Asparaginase

Asparaginase described in the Novozymes dossier catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The enzyme also acts on glutamine but has no activity on other amino acids and asparagine residues in peptides and proteins. 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 ₂ O = 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. Ammonia subsequently reacts with α -ketoglutarate to form L-glutamic acid. The reaction is catalysed by glutamate dehydrogenase in the presence of NADH, which is oxidized to NAD⁺ with the concomitant loss of absorbance measured at 340 nm. The

asparaginase activity is measured as a rate of NADH consumption under standard conditions (pH=7; 37⁰C). The activity of asparaginase is expressed in ASNU activity units. One ASNU is defined as the amount of asparaginase that produces one micromole of ammonia per minute under standard conditions (pH=7; 37⁰C).

Novozymes assessed the potential allergenicity of asparaginase by comparing its amino acid sequence to the amino acid sequences of known or putative allergens in the SWALL (SWISSPROT AND TrEMBLE) and GeneBank databases. It has been reported that an immunologically significant sequence identity requires a match of at least eight contiguous amino acids (Metcalf *et al.*, 1996). No amino acid sequence identity between asparaginase and known or putative allergens was detected at the level of 7 and 8 contiguous amino acids. The amino acid sequence of asparaginase was also assessed for sequence homology with protein sequences that contained the word “toxin” in any of the description fields in the SWALL and GeneBank databases. No significant sequence homology was detected.

Asparaginases are produced by a number of microorganisms, including bacteria and fungi. In many of these microorganisms, two forms of the enzyme are produced, a cytoplasmic and a periplasmic or extracellular asparaginase. The *A. oryzae* asparaginase is an extracellular enzyme.

4.2. Asparaginase enzyme preparation

The asparaginase enzyme preparation is marketed under the name Acrylaway[®] L. The typical composition of the asparaginase enzyme preparation is (approximately):

Total organic solids (TOS):	4%
Water:	46%
Glycerol:	50%
Sodium benzoate:	0.3%
Potassium sorbate:	0.1%

TOS is defined as:

$TOS (\%) = 100 - (A + W + D)$, where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients (JECFA, 2006). The typical asparaginase activity of Acrylaway[®] L is 3500 ASNU/g.

The asparaginase enzyme preparation conforms to the General Specifications for Enzyme Preparations Used in Food Processing (JECFA, 2006). It does not contain significant levels of side activities because the genes encoding extracellular amylases and proteases were deleted from the genome of the asparaginase production strain. The enzyme preparation does not contain viable cells of the production organism.

5. Functional uses

Asparaginase is intended for use as a processing aid during food manufacture to convert asparagine to aspartic acid in order to reduce the formation of acrylamide. Acrylamide is formed from asparagine and reducing sugars primarily in starchy foods that are baked or fried at temperatures above 120⁰C. The reaction between sugars and asparagine proceeds through several

intermediates with acrylamide as the final product formed from the side chain of asparagine. The formation of acrylamide may be reduced by removal of asparagine *via* enzymatic conversion with asparaginase to aspartic acid. Asparaginase will be primarily used during production of starchy foods that are major sources of dietary acrylamide, such as potato chips, French fries, tortilla chips, and dough-based products, including cookies, crackers, pretzels, crisp bread, and biscuits. Novozymes assessed acrylamide reduction in several foods using different levels of asparaginase. Acrylamide reduction ranged from 40 to 98% depending on the food, asparaginase use level, temperature, and holding time.

Asparaginase will be used in dough-based products at levels from 200 to 2500 ASNU per 1 kg of processed food, corresponding to 6-70 g of the asparaginase enzyme preparation (Acrylaway[®]) per 100 kg of processed food. For production of French fries and potato chips, potato strips or slices will be dipped in an asparaginase bath containing approximately 12,000 ASNU/L of water and held for a specified time. Novozymes estimates that the enzyme dosage per 1 kg of the final food product would be 1420 ASNU for French fries and 1820 ASNU for potato chips. These figures are based on the assumption that the maximum water pick-up by potato strips and slices would be 5% and that 1 kg of raw potato strips or slices would yield 0.42 kg of French fries or 0.33 kg of potato chips.

6. Reactions and fate in food

Asparaginase will be used during food processing prior to heat treatment. The enzyme would be subsequently inactivated during heat processing, such as cooking, baking, or frying. As a result of the catalytic activity of asparaginase, low levels of aspartic acid and ammonia are expected to be formed in food. Asparaginase can also hydrolyze free glutamine with the formation of glutamic acid.

7. References

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