FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

SUPPORTING DOCUMENT 1

RISK ASSESSMENT REPORT (Approval)

Executive Summary

Background

Application A1057 seeks approval for the use of an endo-protease as a processing aid. The endo-protease is produced by a genetically modified (GM) strain of *Aspergillus niger* containing additional copies of the endo-protease gene from *A. niger*. The intended technological function of this endo-protease is to reduce haze formation in beer during cold storage.

The risk assessment has considered the technological suitability of the enzyme, the potential hazard of the production organism, as well as assessing the potential hazard of the enzyme preparation. Based on the available data, no food safety concerns, which would preclude permitting its use as a food processing aid, have been identified with the enzyme, or with the microorganism used to produce the enzyme. The absence of identifiable hazards is consistent with the enzyme undergoing normal proteolytic digestion in the gastrointestinal tract. The Application provides adequate information to demonstrate that the enzyme is technologically justified and effective in achieving its stated purpose.

The available data are sufficient to provide confidence in the safety and suitability of the enzyme.

Conclusions

- *A. niger* as the host organism is a well-characterised expression system for the production of enzymes, and has a long history of safe use.
- There was no evidence of systemic toxicity associated with the enzyme preparation following repeat dose (sub-acute and sub-chronic) testing in rats. The No Observed Adverse Effect Level (NOAEL) was 20000 mg/kg bw/day (5040 mg Total Organic Solids /kg bw/day), the highest dose level tested.
- There was no evidence of genotoxicity.
- Based on the reviewed toxicological data it was concluded that, in the absence of any identifiable hazard, an ADI (Acceptable Daily Intake) 'not specified' is appropriate.
- Based on the available evidence, this endo-protease produced in *A. niger* is considered safe for use in foods for human consumption.
- The stated purpose for this endo-protease is to reduce haze formation in beer during cold storage. When used in the form and amounts prescribed, the enzyme is technologically justified and achieves its stated purpose.
- The enzyme meets international purity specifications.

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1. Introduction

An application, received from DSM Food Specialties, seeks approval for the use of an endo-protease as an enzyme processing aid. The enzyme is produced from a genetically modified (GM) strain of *Aspergillus niger* containing additional copies of the endo-protease gene from *A. niger.* There are no permissions for this endo-protease in the Code.

The enzyme is intended for use in beer to reduce or prevent the formation of haze during cold storage due to interaction between polyphenol and haze-active proteins. The endo-protease specifically hydrolyses the haze-active proteins in the beer which effectively prevents complex formation with polyphenols, thus reducing haze formation. The Applicant claims this represents a more cost-effective and energy efficient means of reducing haze formation in beer than the traditional methods of cold stabilisation and filtration.

1.1 Objectives of the Assessment

In proposing to amend the Code to include this endo-protease, derived from a GM strain of *A. niger,* as a processing aid, a pre-market assessment is required.

The objectives of this risk assessment are to determine the following:

- whether any potential public health and safety concerns may arise from the use of endo-protease as a processing aid;
- whether the proposed purpose is clearly stated and the enzyme achieves its technological function in the quantity and form to be added.

1.2 Risk Assessment Questions

The following risk assessment questions have been developed to address the objectives of the assessment:

- Does the enzyme preparation present any food safety issues?
- Does the enzyme achieve its stated technological purpose?

2. Characterisation of the endo-protease

2.1 Identity of the enzyme

The following information regarding the identity of the enzyme has been taken from the Application and verified from enzyme nomenclature references.

Systematic name:	Prolyl oligopeptidase
IUBMB Enzyme nomenclature:	EC 3.4.21.26[1]
C.A.S. number:	9001-92-7
Common name:	endo-protease
Other names:	propyl endopeptidase, proline endopeptidase, post-proline cleaving enzyme, proline-specific endopeptidase, post-proline endopeptidase, endopropylpeptidase,
Marketing name:	Brewers Clarex

2.2 Chemical and physical properties

2.2.1 Enzymatic properties

The function of the enzyme is the hydrolysis of proline, and to a lesser extent, alanine in oligopeptides. The Application states that the enzyme hydrolyses peptides at the carboxyl site of proline residues to produce smaller peptides with a proline residue at the C-terminus of one of the two smaller peptides, (or a peptide plus the amino acid proline) and amino acids.

The molecular weight of the enzyme is 56 kDa (deduced from the amino acid sequence) or 66 kDa (from SDS-PAGE analysis). The larger apparent molecular weight on SDS-PAGE is due to glycosylation of the enzyme.

The optimum pH for using the enzyme is 4.6, with approximately 60% efficiency at pH 5.5. The optimum temperature for enzyme activity is around 50°C. The enzyme is quite stable at this temperature, with about 90% activity after 10 hours at 50°C and pH 5. However, the enzyme activity reduces above 50°C, and is completely inactivated at beer pasteurisation conditions.

The Applicant has an in-house assay method for determining enzyme activity. The activity is expressed in so-called Propyl Peptidase Units (PPU). One PPU is defined by the Applicant as the quantity of enzyme that will liberate p-nitroanilide at a rate of 1 µmol per minute under the conditions of the assay (pH 4.6 and 37°C). More details about the enzyme activity assay are provided in the Application.

The endo-protease enzyme preparation may also have alpha-amylase side activity.

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[1] The Applicant originally proposed the EC number to be listed as 3.4.21.x, but FSANZ is unable to approve an enzyme without a complete EC number. Following discussion with the Applicant it was agreed that 3.4.21.26 would be the appropriate EC number.

2.2.2 Physical properties

The commercial enzyme preparation is a light brown to brown liquid with a pH range of 3.8-4.2 and typically has an enzyme activity of 5.0-5.8 PPU/g. The preparation is formulated with glycerol to ensure the desired and standardised activity concentration is achieved.

2.3 Production of the enzyme

2.3.1 Fermentation process

The endo-protease enzyme preparation is produced using standard commercial enzyme production techniques. It is produced using a submerged fermentation of a selected pure culture of the source organism, *A. niger.* Discussion of the production and description of the source organism is provided in later sections.

The production steps can be summarised as a fermentation process, recovery steps to extract the enzyme from the fermentation broth, purification steps and then formulation of the final commercial enzyme preparation.

The fermentation process involves two steps, being the initial inoculum fermentations to produce enough of the microorganism for the production fermentation, and then the main fermentation.

The downstream processing steps taken after the main fermentation to produce the enzyme consist of: killing the production strain (source microorganism), removal of the cell material, ultrafiltration to separate and concentrate the enzyme and finally stabilisation and standardisation using glycerol to produce the desired enzyme activity in the final enzyme preparation.

2.3.2 Description of the genetic modification

Derivation of the Host Strain

The recipient organism for the genetic modification is a glucoamylase and protease negative *A.niger* strain designated ISO-508. Strain ISO-508 was derived from the DSM *A. niger* strain GAM-53[2] by genetic modification. The GAM-53 strain was derived by classical mutagenesis from the original *A. niger* strain NRRL 3122, which has been in use for the production of glucoamylase since the 1960s (van Dijck et al 2003).

GAM-53 was originally selected in 1982 for its enhanced production of the enzyme glucoamylase. Subsequent analysis of the strain showed that the enhanced production of glucoamylase was due, in part, to an increase in a number of gene copies in the strain. Whereas the original NRRL3122 strain only contained one copy of the glucoamylase (*glaA*) gene, the GAM-53 strain contains seven copies of *glaA*. GAM-53 has subsequently been used as a host for the random integration and over-expression of genes for enzymes such as phytase and xylanase and more recently has been genetically modified to generate new strains which can serve as recipients for the targeted integration and over-expression of genes of interest (van Dijck et al 2003). This has been achieved by deleting the seven *glaA* loci, creating so-called *ΔglaA* loci, which can then serve as sites (called 'plug-sites') for targeted integration of genes encoding various enzymes of interest.

[2] Stored in the DSM Culture Collection as DS 3043.

Following creation of the 'plug-sites', the gene encoding the major protease (*pepA*) was inactivated using recombinant-DNA techniques and the strain's capacity to secrete proteins was improved using classical mutagenesis and selection. The resulting recipient strain ISO-508 was then used to construct the endo-protease production strain GEP-44.

Construction of the Production strain

Two gene expression cassettes were used to construct the production strain: one containing the endo-protease gene (*gepA*) from *Aspergillus niger* and the other encoding the selectable marker gene *amdS* encoding acetamidase from *Aspergillus nidulans*. The endo-protease and acetamidase expression cassettes were cloned into the *Escherichia coli* vector pTZ18R generating the plasmids pGBTOPGEP-1 and pGBAAS-1, respectively.

The endo-protease gene is under the control of the *glaA* promoter and flanked with sequences from the 3' end of the *glaA* gene and the acetamidase gene is under the control of the *gpdA* (glyceraldehyde-3-phosphate dehydrogenase gene) promoter from *A. nidulans* and also flanked by sequences from the 3' end of the *glaA* gene. The flanking 3' glaA sequences facilitate targeted integration (via homologous recombination) of the *gepA* and *amdS* genes into one of the seven *ΔglaA* loci ('plug-sites').

The endo-protease and selectable marker expression cassettes, with all *E. coli* vector DNA sequences removed, were integrated into the chromosome of ISO-508 by co-transformation. Transformants were selected for their ability to use acetamide as a sole carbon source. Acetamide positive transformants were further analysed using polymerase chain reaction (PCR) for the presence of multiple copies of the endo-protease expression cassette. Counter-selection on fluoro-acetamide was then used to select a natural variant which had the *amdS* selectable marker deleted as a result of a natural recombination event. The absence of the *amdS* marker was confirmed by Southern analysis. The resulting isolate was thus free of any vector sequences derived from *E. coli* as well as the *amdS* selectable marker gene.

The region comprising the *gepA* gene was then multiplied into the other *ΔglaA* loci by gene conversion (Selten et al 1998), a natural spontaneous recombination event which does not involve mutagenic treatment. Strains that had an increase in the number of filled *ΔglaA* loci were identified using DNA gel electrophoresis. A strain was chosen that contained sufficient gene copies to allow for commercially attractive expression levels of the endo-protease enzyme: this strain was designated GEP-44.

Genetic stability of the source organism

Strains belonging to the *A. niger* GAM-lineage are considered to be genetically stable strains, having been stably cultured and stored for more than 30 years by the Applicant. The Applicant states that new cultures are routinely tested for stability in relation to particular morphological, growth, production and product characteristics. Occasionally, morphologically dissimilar colonies are observed following plating out but this is not unusual, and the cultures remain stable in every other aspect.

In relation to the production strain (GEP44) itself, the Applicant reports that its stability in terms of behaviour in strain management and enzyme production characteristics is not dissimilar to the parental GAM strains. Furthermore, since the endo-protease expression cassette is integrated into the chromosome and also does not contain any extraneous sequences from the *E. coli* cloning vector (such as the origin of replication), it is unlikely that the introduced endo-protease gene would be transferred horizontally to another, unrelated organism.

2.4 Analysis and Specifications

2.4.1 Methods of analysis

The Applicant has an in-house method of analysis for determining the activity of the enzyme of the enzyme preparation, which is contained in the Application.

A method of analysis for the presence of the enzyme or source organism in treated food is unnecessary. This is because the enzyme is inactivated during the heating step in the brewing process, and there are no residues of the source organism in the enzyme preparation, so none will be remaining in the final food.

2.4.2 Specifications

There are international specifications for enzyme preparations used in the production of food which have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) (monograph 3, 2006) and the Food Chemicals Codex (7th edition, 2010). The specifications for this endo-protease meet these specifications. Both of these specifications are primary reference sources for specifications listed in clause 2 of Standard 1.3.4 – Identity and Purity, of the Code.

Table 1: *Specifications for three representative samples of commercial endo-protease preparations compared to JECFA specifications for enzymes*

Analysis	Sample 1	Sample 2	Sample 3	JECFA spec
Lead (mg/kg)	<0.2	<0.2	<0.2	≤ 5
Arsenic (mg/kg)	<0.02	<0.02	<0.04	-
Mercury (mg/kg)	<0.02	<0.02	<0.4	-
Cadmium (mg/kg)	<0.01	<0.01	<0.02	-
Total plate counts (cfu/ml)	40	20	<100	-
Coliforms (cfu/ml)	<10	<10	<10	≤30
Salmonella (absent in 25 ml)	Absent	Absent	Absent	Absent
E. coli (absent in 25 ml)	Absent	Absent	Absent	Absent

The Application states that the endo-protease preparation contains no antimicrobial activity, as also required by the JECFA specifications for enzymes used in food processing. The Applicant confirmed that there are no mycotoxins found in the enzyme preparations.

It is also important for the Applicant, for proprietary commercial reasons, that the final commercial enzyme preparation does not contain any viable production organisms. The cells are killed off, using benzoate, and ultrafiltration and sterile filtration is performed during the final clean up and purification step to remove any residual dead cells and organisms from the enzyme preparation.

The final enzyme preparation meets international specifications for enzyme preparations used in the production of food.

The enzyme preparation does not contain any allergenic substances that would require mandatory labelling declarations.

3. Technological function of the enzyme

The technological function proposed by the Applicant is to use the endo-protease, to treat beer during manufacture to reduce the formation of haze in the final packaged beer during cold storage, so-called chill-haze formation. Chill-haze is due to the formation of visible haze particles formed from reactions of polyphenols and haze-active proteins to form larger chemical complexes. Both the polyphenols and haze-active proteins are present as components in beer being extracted from the ingredients used to produce beer (mainly derived from malt and hops) (Asano et al., 1982).

Traditionally, beer producers have a cold stabilisation step in their production process to first chill the beer and maintain it at this cold temperature for a period of time to allow the formation of chill haze particles. The haze particles form due to the interaction and binding to each other of polyphenols and haze-active proteins (specifically containing proline amino acids) (Edens et al. 2005 and Lopez and Edens 2005). The haze particles are removed from the beer (along with other particulates, such as dead yeast cells and carbohydrate complexes) by a cold beer filtration step before the clear beer is packaged.

Brewers also use other treatments to improve the colloidal stability of the beer by reducing the concentration of polyphenols (e.g. using PVPP, polyvinyl polypyrrolidone) and/or haze-active proteins (e.g. using silica gels of different types) (Edens et al. 2005 and Lopez and Edens 2005).

An alternative or complementary treatment is to use different types of enzymes during the manufacture of beer to hydrolyse proteins, so reducing their size and the size of resulting polyphenol-haze-active proteins complexes so they are not visible as haze. An example of such an enzyme treatment is the well-known and used papain enzyme. However none of the enzymatic activities present in papain are able to hydrolyse and so reduce the size of high proline proteins, which are the proteins implicated in the formation of chill haze (Asano et al. 1982).

The use of this endo-protease has recently been developed and commercialised as an alternative beer processing treatment that also reduces the formation of chill haze (Edens et al. 2005 and Lopez and Edens 2005).

This endo-protease is a proteolytic enzyme that specifically hydrolyses proline-rich proteins and so reduces their size. This limits the size of any polyphenol/haze-active protein complexes that are formed. Such smaller complexes that may be produced from the reactions between polyphenols and smaller protein fragments are either still soluble in cold beer or small enough not to produce visible haze. Laboratory trials and then larger scale production trials reported in the journal articles submitted with the Application concluded that endo-protease treated beer had improved chill haze colloidal stability compared to control beers and also comparable results to those obtained by traditional treatments using other stabilisation treatments such as PVPP and/or silica gel.

The Applicant and the researchers dose the endo-protease (stated to be 2 mL of the enzyme preparation to 100 L of the wort) at the start of beer fermentation which is early in the beer production process. The treated beer undergoes the standard beer processing conditions including maturation and cold stabilisation storage, though it is claimed this storage time can be reduced. The stabilised beer is cold filtered to remove haze material and particulates and then packaged (Edens et al. 2005 and Lopez and Edens 2005). Packaged beer normally undergoes a pasteurisation step which inactivates the enzyme due to the time and temperature of the pasteurisation process.

The Applicant claims that other specific quality parameters critical for producing commercial beer such as flavour and formation and stability of beer foam, are not affected by the endo-protease treatment.

3.1 Conclusion

The technological function of the endo-protease enzyme as stated by the Applicant has been demonstrated. That is, this endo-protease is effective as an alternative beer treatment agent to reduce the formation of chill haze in final package beer.

4. Hazard Assessment

The hazard of the endo-protease preparation was assessed by:

(1) considering the donor/host organism, including its history of safe use in food production processes;

(2) evaluating unpublished toxicity studies using the endo-protease preparation.

4.1 Hazard of the donor/host organisms

The taxonomic identity of the recipient strain (GAM53) and the production strain (GEP44) have been confirmed as *Aspergillus niger* v. Tieghem. The endo-protease is produced from a genetically modified (GM) strain of *A. niger* which has been obtained through self-cloning. This means that *A. niger* is both the source (donor) of the introduced gene as well as the host (recipient).

Aspergillus niger is a member of the genus *Aspergillus* which consists of over 260 species of fungi that are generally considered asexual, although forms that reproduce sexually have also been found. Aspergilli are ubiquitous in nature. They are geographically widely distributed and have been observed in a broad range of habitats. *A. niger* is both a species and a group within the genus *Aspergillus*.

A. niger is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles and other decaying vegetation. The spores are widespread and are often associated with organic materials and soil.

A. niger has been used for several decades for the commercial production of organic acids and various food enzymes and is generally regarded as having a history of safe use and is not considered to be a significant human pathogen (EPA 1997). While certain strains of *A. niger* appear to be capable of producing mycotoxins, there is no evidence that any of the industrial strains used have produced detectable levels of such toxins under the fermentation conditions used (van Dijck et al 2003).

Strains from the GAM lineage of *A. niger*, including those that have been genetically modified using both random and targeted integration techniques, have also been extensively tested for their ability to synthesise mycotoxins and other secondary metabolites (e.g. nigragillin and naphtha-γ-pyrones) under fermentation conditions optimal for their production. In no circumstances have any mycotoxins or other secondary metabolites been detected either in broth samples or in the final enzyme preparation (van Dijck et al 2002, van Dijck et al 2003).

Additionally, culture extract from the GEP44 production organism as well as two samples of fermentation broth (straight broth plus an ultra filtrated concentrate) were analysed for the presence of toxic metabolites. The GEP44 strain was cultured on media which is known to be optimal for expression of fungal secondary metabolites and then analysed by HPLC with diode array detection. The samples of fermentation broth were similarly analysed. Identified metabolites were compared to a spectral UV library made from standards of all important fungal toxins. The GEP44 strain was shown to produce several unknown metabolites, but no known toxic metabolites were found. In samples of the fermentation broth ergosterol was found but no mycotoxins. Ergosterol is a biological precursor to vitamin D_2 and is a natural component of fungal cell membranes. Its presence does not represent a concern.

4.2 Evaluation of unpublished toxicity studies

Unpublished toxicity studies on the endo-protease protein were submitted by the Applicant and independently evaluated by FSANZ. These studies included sub-acute (14-day) and sub-chronic oral toxicity studies in rats, and two *in vitro* genotoxicity assays. All toxicity studies were done using the same enzyme preparation (JLL 03 006 IDF) which was produced according to the normal commercial production method. The enzyme preparation had an activity of 11.0 PPU/g and a TOS[3] content of 25.2%.

All toxicity studies were performed in compliance with the OECD Principles of Good Laboratory Practice (GLP) and all except the sub-acute (14-day) study were performed according to the relevant OECD Test Guideline.

4.2.1 Sub-acute toxicity study

Satish PM (2003) Repeated dose (14-day) oral toxicity study by gavage with enzyme preparation of *Aspergillus niger* (GEP44) in Wistar rats. Study No. 3715/03. Lab: Rallis Research Centre, India. Sponsor: DSM Food Specialties, Netherlands. **GLP**: OECD. **QA Statement**: Yes. **Test Guidelines**: OECD Test Guideline 407 with modifications.

The purpose of the 14-day study was to assess the systemic toxic potential of the enzyme preparation when administered by gavage to rats and also to provide information for selection of dose levels for a subsequent sub-chronic study. The study was conducted as per OECD Test Guideline 407 ("Repeated Dose 28-Day Oral Toxicity Study in Rodents") but was modified to a 14-day pre-study which did not include recovery groups for the control and high dose groups. Haematology, clinical chemistry and histopathology were also not done.

GEP44 was administered by oral gavage to male and female Wistar rats (6/sex/group) at doses of 2000, 7000 and 20000 mg/kg bw/day in double distilled water for 14 days. The control group received double distilled water only. The dose volume was 20 ml/kg bw except for the high dose group which received 18.54 ml/kg bw. The rats were aged 7 weeks at the start of treatment and weighed 191-220 g for males and 131-170 g for females.

It was not specified in the study report whether rats were fasted prior to dosing. Food and water were available *ad libitum* following dosing. Veterinary examination was carried out prior to dosing and at weekly intervals. Rats were observed for clinical signs daily and for pre-terminal deaths twice daily. Individual body weights were recorded on day 1, 5, 8, 12 and 15. Survivors were killed at the end of the observation period after overnight fasting and necropsied.

[3] Total Organic Solids

There were no deaths. No clinical signs were observed during the study. Some hair thinning was observed in the control, low and mid dose groups but was considered incidental to treatment. No significant changes were observed in mean body weights and in cumulative net weight gains in any group nor was any change in food consumption observed for any of the groups. There were no treatment-related macroscopic abnormalities. The No Observed Adverse Effect Level (NOAEL) was 20000 mg/kg bw/day (5040 mg TOS/kg bw/day), equivalent to the highest dose level tested.

4.2.2 Sub-chronic toxicity study

Krishnappa H (2003) Repeated dose 90-day oral toxicity study by gavage with enzyme preparation of Aspergillus niger (GEP44) in Wistar rats. Study No. 3716/03. Lab: Rallis Research Centre, India. Sponsor: DSM Food Specialties, Netherlands. **GLP**: OECD. **QA Statement**: Yes. **Test Guidelines**: OECD Test Guideline 408.

GEP44 was administered by oral gavage to male and female Wistar rats (10/sex/group) at doses of 2000, 7000 and 20000 mg/kg bw/day for 90 days. The vehicle was double distilled water and the dose volume was 20 ml/kg bw, except for the high dose group where the dose volume was 18.54 ml/kg bw. The control group received vehicle only. Rats were 7 weeks old and had a bodyweight range of 181-210 g for males and 141-170 g for females at dosing. Food and water were available *ad libitum*.

Clinical signs were recorded daily, with observations for morbidity and pre-terminal death done twice daily. A detailed clinical/veterinary evaluation was performed weekly. Body weight and food consumption were recorded weekly. Ophthalmological examination was performed on all rats one day prior to treatment and at the end of the treatment period. A detailed neurological examination was conducted at the end of the treatment period. Blood samples were collected at termination for analysis of standard haematology and clinical chemistry parameters. At the end of the treatment period, survivors were killed and necropsied. Standard organs were weighed and those from the control and high dose groups were histopathologically examined, along with any organs showing lesions from the low and mid dose groups. The lungs from animals in the low and mid dose groups were also histopathologically examined for evidence of infection to provide an assessment of the health status of the animals.

There were no deaths during the study, nor were any treatment-related clinical signs or effects on bodyweight observed in any of the groups. Significantly lower food intake was observed in males on week 1 at mid and high doses and on weeks 8 to 13 (except week 12) at high dose compared to the control but were not associated with any bodyweight changes. The decrease in food intake was therefore not considered to be toxicologically relevant but rather an adaptation to the increased energy intake via the test material. For females, mean body weights and cumulative net body weight gains were higher throughout the treatment period in the high dose group, achieving statistical significance on weeks 6 and 8-10 for mean body weight and weeks 4-13 for cumulative net body weight gains. Food intake was significantly lower in week 1 in the high and mid dose groups. These differences were not considered to be toxicologically relevant but rather due to increased energy intake via the test material. Subsequent calculations confirmed that the extra energy in the diet supplied by the test material would account for the observed weight gain in the animals. No treatment-related neurological effects were observed in any of the groups. Incidences of lower hind limb foot splay values were observed for mid and high dose male and females, but no significant effects were observed in related end points (grip strength and motor activity), nor was there any dose-relationship or other functional alterations. These findings were therefore considered to be incidental and not treatment related. No eye abnormalities were observed in any of the treatment groups.

There was no treatment-related effect on any haematology or clinical chemistry parameter. Some small differences were observed for various parameters but no dose response relationship was evident and the values were within the range of historical control data. There were no treatment-related macroscopic or microscopic findings. The changes observed represented common background pathology findings in rats of this strain and age and occurred in one or a few animals only or were randomly distributed among the groups. The No Observed Adverse Effect Level (NOAEL) was 20000 mg /kg bw/day (5040 mg TOS/kg bw/day), equivalent to the highest dose level tested.

4.2.3 Genotoxicity

Two *in vitro* genotoxicity studies were submitted as part of the current Application (Table 2). These studies were GLP compliant and conducted according to appropriate test guidelines. Signed QA statements were contained in the respective study reports. The two *in vitro* studies were conducted in the presence and absence of an exogenous source of metabolic activation (S9 liver preparations from Aroclor 1254-induced rats). Positive and negative (vehicle) controls were tested in each study and gave expected results. The enzyme preparation showed no evidence of mutagenic or clastogenic activity in these assays.

Table 2: *Summary of genotoxicity studies*

Test	System	Test Material	Conc./Dose	Results
Bacterial reverse mutation (Ames) test (Krul 2003)	*Salmonella typhimurium* strains TA98, TA100, TA1535 & TA1537 *Escherichia coli* strain Wp2uvrA (±S9)	GEP44 (Lot No. JLL03006IDF) Vehicle was milli-Q water	Assay 1: 62-5000 µg/plate Assay 2: 312-5000 µg/plate	Presence of false positives in Assay 1[4] Assay 2 used protocol to exclude false positive results – no increase in the number of observed revertants No precipitation or cytotoxicity observed
Mammalian chromosomal aberration test (de Vogel 2003)	Cultured human peripheral lymphocytes (±S9)	GEP44 (Lot No. JLL03006IDF) Vehicle was RPMI-1640 growth medium without serum	0-5000 µg/ml	Not clastogenic for cultured human lymphocytes

4.3 JECFA Consideration

JECFA has not evaluated this endo-protease from *A. niger*.

4.4 Conclusions

There are no public health and safety issues associated with the use of endo-protease (GEP44) as a food processing aid on the basis of the following considerations:

- *A. niger* is a well-characterised expression system for the production of enzymes, and has a long history of safe use.

[4] False positives due to the presence of histidine in the preparation

- There was no evidence of systemic toxicity associated with the enzyme preparation following repeat dose (sub-acute and sub-chronic) testing in rats. The NOAEL was 20000 mg/kg bw/day (5040 mg TOS/kg bw/day), the highest dose level tested.

- The enzyme preparation was not genotoxic *in vitro*.

Based on the absence of toxicity of the endo-protease preparation, as well as the absence of toxigenic potential of the host organism, an ADI 'not specified' is considered appropriate.

5. Dietary Exposure

Processing aids perform their technological function during the manufacture of food and are not active in the final food. They are usually used at low levels, sufficient to achieve the purpose. Enzymes functioning as processing aids are usually removed or inactivated during further processing of the food. No endo-protease activity can be detected following pasteurisation of the beer. Given the absence of any detectable enzyme activity, any residual enzyme would be expected to be present as denatured protein and would undergo normal proteolytic digestion in the gastrointestinal tract.

Based on calculations provided by the Applicant, the inactivated enzyme remains inert in the final food at a concentration of 15 mg TOS/L beer. Based on beer consumption data for the Netherlands, the Applicant calculated that a 60 kg person consuming beer at the 90th percentile would have an estimated daily intake of inactivated enzyme of 1.25 mg TOS/kg bw/day. The NOAEL of 5040 mg TOS/kg bw/day therefore provides a very large margin of safety. This large margin of safety, which would also be expected based on an Australian/New Zealand diet, combined with the allocation of an ADI "not specified" indicate that further dietary exposure assessment is unnecessary.

6. Response to Risk Assessment Questions

Does the enzyme preparation present any food safety issues?

The Hazard Assessment reviewed evidence examining potential toxicity and genotoxicity associated with the enzyme preparation. There were no hazards identified which would preclude permitting the use of the enzyme as a food processing aid. The presence of residual amounts of inactivated endo-protease in the final food does not represent a safety concern. Any enzyme residue remaining in the beer would be expected to undergo normal proteolytic digestion in the gastrointestinal tract.

Does the enzyme achieve its stated technological purpose?

The Application clearly articulates the stated purpose for this enzyme, namely for the hydrolysis of haze-active proteins in the beer which effectively prevents complex formation with polyphenols, thus reducing haze formation. The evidence submitted in support of the Application provides adequate assurance that the endo-protease, in the form and amounts added, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

7. Conclusion

The risk assessment has considered the technological suitability, the potential hazard and identity of the donor/host microorganism and the potential hazard of the endo-protease enzyme preparation.

The evidence presented was sufficient to determine that no safety concerns with the enzyme or donor/host microorganism exist. Thus endo-protease is unlikely to pose any health risk when used as a food processing aid. It was further concluded that the proposed use of the enzyme, namely as a processing aid to prevent haze formation in beer during cold storage, was technologically justified in the form and prescribed amounts, and demonstrated to be effective.

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