APPLICATION FOR THE AMENDMENT OF THE AUSTRALIA / NEW ZEALAND FOOD STANDARDS CODE STANDARD 1.3.3 WITH ENDO-PROTEASE FROM A SELFCLONED STRAIN OF *ASPERGILLUS NIGER*

DSM 000584-001

EXECUTIVE SUMMARY

DSM Food Specialties (“DSM”) manufactures endo-protease, which is produced by submerged fermentation of a selected, pure culture of an *Aspergillus niger* expressing the endo-protease gene from *Aspergillus niger*. DSM produces the endo-protease preparations in liquid form. It is standardized with glycerol. The trade name will be Brewers Clarex.

The endo-protease will be used in beer where its technological function is to reduce the haze-forming after production, leading to decreased processing costs for the beer producer.

In this dossier the application of the endo-protease enzyme produced with a selfcloned *Aspergillus niger* strain is described. The dossier has been written in the format of the Food Standards Australia New Zealand, Application Handbook, issue 1 July 2010. The application is related to the Standards for food production and is addressed to the information requirements of Section 3.1 (General requirements) and Sub-section 3.3.2 (Processing aids).

Purpose of this application is to amend Standard 1.3.3. Processing Aids, section 17 by the addition of endo-protease (EC 3.4.21.x) produced by an *Aspergillus niger* strain containing the gene for endo-protease from *Aspergillus niger*.

The amendment is supported in this dossier by:

- Section I, containing general information
- Section II, containing specific information on endo-protease as a processing aid, consisting of:
    - Section A with technical information on the processing aid
    - Section C with information on the safety of the processing aid including the results of toxicological studies and a conclusion and margin of safety; the conclusion is that the processing was proved to be safe by studies and tests.
    - Section D with additional information on the microbiological issues including safety and stability aspects of the source micro-organism
    - Section E with additional information of the safety aspects concerning the genetic modification of the micro-organism
    - Section F with information of the dietary exposure to the processing aid
    - A list of relevant annexes and references in respectively the Sections G and H
- Section III, containing a checklist according to the Application Handbook, Section 3.1.11 (General requirements).

Remarks:

The electronic version of this dossier has been divided over the following files:

- Registration dossier endo-protease
- 22 Annexes, each containing 1 or more files
- 20 References

I GENERAL INTRODUCTION

1 Applicant details

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Nature applicant

DSM Food Specialties develops, produces and sells a broad spectrum of ingredients for the food industry.

Manufacturer

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2 Purpose of the application

It is the intention of DSM Food Specialties, The Netherlands to submit an application for the use of a new processing aid. This processing aid is an enzyme, more particular endo-protease produced by a selfcloned microbial strain, *Aspergillus niger*.

The enzyme is not yet approved by FSANZ although several other peptidases, produced by several microbial sources are.

Purpose of this application is to amend Standard 1.3.3. Processing Aids, table to clause 17([1]) as follows:

Endo-protease EC 3.4.21.x	*Aspergillus niger*

This application is made on behalf of the applicant DSM Food Specialties B.V.

Applications for the use of endo-protease from *Aspergillus niger* have already been filed successfully in France, Russia, Denmark and China. In the USA protease from *Aspergillus niger* has been notified as GRAS (GRN 000089).

3 Justification for the application

3.A. Need and/or advantages for the proposed change

By this change of the code, brewers can choose for another way to prevent haze in the final product. Using this enzyme as processing aid is more cost effective and environmental friendly than the traditional cooling and filtering steps are.

3.B.1 The safety of the processing aid

The processing aid is safe as described in section II.C.

3.B.2 Nutritional issues related to the proposed change

Given the very small amount of enzyme to be added to the beer (4 ml enzyme preparation per hl beer), no relevant nutritional effects are foreseen.

3.C Technological need for the processing aid

The technological function of the endo-protease in beer is to prevent the appearance of haze during (cold) storage due to polyphenol – haze-active protein interactions.

In the course of time the haze-active proteins and polyphenols which are both present in the beer, complex with each other (coupling of the proline imino acids of the haze active proteins with the polyphenols) to form a haze. In conventional production, beer producers have a cold stabilization step in their process in order to facilitate the formation of this haze, which can then be removed by a filtration step, before packing the beer. These steps are both time- and energy- consuming. Additionally, to colloidally stabilize the beer over a longer period of time (i.e. its shelf life period), they can use silica gels and/or (regenerable) PVPP[2] in order to remove the haze-active proteins and the polyphenols, repectively. By taking out either the haze-active proteins, or the

[1]) In the Handbook called clause (o)

[2] Polyvinylpolypyrrolidone

polyphenols, or both, the complex formation between the two (i.e. haze formation) is prevented during the beer shelf life. When endo-protease is used, the (proline-rich) haze-active-proteins are specifically hydrolysed and thereby complex formation between proteins and polyphenols is effectively prevented. In addition, since the complex formation between proteins and polyphenols is already prevented during the fermentation stage, where the endo-protease hydrolyses the haze-active proteins, the cold stabilization step can be significantly reduced in time or operated at higher temperatures. In this way the cold-stabilization capacity increases for the brewers without the need of capital expenditure while brewers can save energy in the cooling process (both higher temperature and shorter duration), thus saving money, energy and the environment.

3.D. Potential impact on trade

The total impact of the use of this enzyme as processing aid will be relatively small, as the volume of enzyme preparation to be used is only a few ml per hl beer. It will however affect trade volumes of both silica-gel and PVPP.

3.E. Consumer choice

Consumers will not notice the difference between beers produced the current standard way or those produced with this enzyme. There will be no taste differences in the beer after being produced with either method.

3.F. Interest of the industry

The enzyme is already sold to several breweries in other parts of the world and also Australian breweries have shown interest.

3.G. The costs and benefits for industry, consumers and government associated with use of the processing aid.

Beer prepared with DSM's endo-protease will be more cost effective and more environment-friendly to produce. Brewers Clarex thus allows significant savings for the industry. By applying Brewers Clarex, brewers can achieve a reduction in carbon foot-print. The benefits for consumers lie in the reduction of the carbon footprint as mentioned above. As mentioned before, the introduction of this enzyme will have a beneficial effect on the environment because of the elimination of the energy-consuming cooling step, thereby underlining the government's effort to reduce carbon emission.

No additional costs are associated with the use of endo-protease for industry, consumers and the government.

4 Information to support the application

The safety of the product is discussed in section II.D; over the last 12 months significant sales took place in other countries by several globally operating companies. Companies

are considering further implementation of Brewers Clarex around the world, including Australia and New Zealand.

5 Assessment procedure

According to DSM, this dossier should be assessed according the General Procedure, level 1 since it applies for the allowance of an enzyme as processing aid that is already permitted, but not yet in this micro-organism.

6 Confidential commercial information (CCI)

No confidential commercial information is incorporated in this dossier.

7 Exclusive capturable commercial benefit (ECCB)

The declaration of exclusive capturable commercial benefit is available as Annex I.7-1

8 International and other national standards

8.A. International Standards

The enzyme complies with the specifications for enzymes of both JECFA (JECFA, 2006) and the Food Chemicals Codex (FCC, 2010) as explained in Section II.A.5. There is no Codex standard for the enzyme, since there are no specific Codex standards for enzymes.

8.B. Other National Standards or Regulations

Applications for the use of endo-protease from *Aspergillus niger* have already been filed successfully in France, Russia, Denmark and China. In the USA protease from *Aspergillus niger* has been notified as GRAS (GRN 000089).

9 Statutory declaration

The statutory declaration is available as Annex I.9-1

10 Checklist

The checklist is available as Section III

II PROCESSING AIDS

A. Technical information on the processing aid

1. Information on the type of processing aid

Endo-protease is an enzyme from microbiological origin, thus falling in category o.

2. Information on the identity of the processing aid

- Systematic name : prolyl oligopeptidase
- Accepted name : endo-protease
- Other names : peptidase, prolyl endopeptidase, proline endopeptidase , endoprolylpeptidase,
- Marketing name : Brewers Clarex
- IUPAC/IUB Number : EC 3.4.21.x
- CAS number : 9001-92-7
- EINECS number : 232-642-4

Endo-protease belongs to the subclass of peptidases.

The amino acid sequence of the enzyme is as follows:

ATTGEAYFEQLLDHHNPEKGTFSQRYWWSTEYWGGPGSPVVLFTPGEVSADGYEGY
LTNETLTGVYAQEIQGAVILIEHRYWGDSSPYEVLNAETLQYLTLDQAILDMTYFAETVK
LQFDNSTRSNAQNAPWVMVGGSYSGALTAWTESVAPGTFWAYHATSAPVEAIYDYW
QYFYPIQQGMAQNCSKDVSLVAEYVDKIGKNGTAKEQQALKELFGLGAVEHFDDFAAV
LPNGPYLWQDNDFATGYSSFFQFCDAVEGVEAGAAVTPGPEGVGLEKALANYANWFN
STILPDYCASYGYWTDEWSVACFDSYNASSPIYTDTSVGNAVDRQWEWFLCNEPFFY
WQDGAPEGTSTIVPRLVSASYWQRQCPLYFPETNGYTYGSAKGKNAATVNSWTGGW
DMTRNTTRLIWTNGQYDPWRDSGVSSTFRPGGPLASTANEPVQIIPGGFHCSDLYMA
DYYANEGVKKVVDNEVKQIKEWVEEYYA

- Host organism : *Aspergillus niger*
- Donor organism : *Aspergillus niger*

3. Information on the chemical and physical properties of the processing aid

Possible interactions with different foods

The endo-protease from the selfcloned *Aspergillus niger* strain described in this dossier hydrolyses peptides at the carboxyl site of praline residues. The reaction products are 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.

Most of the (malt) proteins and peptides in beer are subject to (enzymatic) degradation during malting/mashing or by yeast during the fermentation stage. This process occurs

by native proteases originated from the malting stage and/or the yeast. So, the hydrolysis of proteins and peptides as such is not a new phenomenon. There is therefore no basis to believe that other interactions of the enzyme have relevance to humans.

Enzymatic properties

The activity of the endo-protease is expressed in so-called Prolyl Peptidase Units (PPU).

One PPU is defined as that quantity of enzyme that will liberate p-nitroanilide at a rate of 1 μmol per minute under the conditions of the assay.

This assay method is shown in Annex II.A.3-1. The method is based on the release of the p-nitroanilide from the synthetic substrate Z-Gly-Pro-pNA[3] at pH 4.6 and 37°C. The formed p-nitroanilide is a measure for the prolyl oligopeptidase activity[4].

The molecular weight (MW) of the enzyme, deduced from the amino acid sequence is 56 kDa and on SDS-PAGE 66 kDa due to glycosylation of the protein.

The enzyme has an optimum on pH 4.6 as measured on Z-Gly-Pro-pNa and has around 60% at pH 5.5.

The temperature optimum of the endo-protease lies around 50°C. The enzyme is stable (10 hours at 50°C, ca. 90% residual activity) at pH 5. Above 50°C the enzyme begins to inactivate. The enzyme is completely inactivated after 5 minutes at 99°C.

Subsidiary enzymatic activities

The enzyme preparation may contain alpha amylase side activity, depending on the downstream processing used. According the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) alpha amylase has the following classification:

- Systematic name:	4-α-D-glucan glucanohydrolase
- Recommended name/synonyms:	alpha amylase , endoamylase
- Enzyme Commission number:	3.2.1.1
- CAS number:	9000-90-2
- EINECS number:	232-565-6

Particle data

The processing aid is not particulate.

[3] Carbobenzoxy-glycine-proline-p-Nitroanilide

[4] The Conversion HUT (fungal protease) activity/PPU activity is ca. 14.2

4. Manufacturing process

DSM's endo-protease is produced by a controlled submerged fermentation of a selected, pure culture of *Aspergillus niger* (see Sections D and E). The production process includes the fermentation process, recovery (downstream processing) and formulation of the product. An overview of the different steps involved is given in Annex II.A.4-1.

Fermentation process

The fermentation process consists of two steps: inoculum fermentations and main fermentation. The whole process is performed in accordance with Good Food Manufacturing Practice (see below).

Inoculum fermentations:

The inoculum fermentations consist of two pre-culture phases. During the first phase, a vial with a pure culture of a strain of the *Aspergillus niger* is suspended in purified water. For seeding, this suspension is aseptically transferred to a fermentor puntbus containing a medium[5] which is sterilized, safe and suitable for food production raw materials

Subsequently, the contents of the first phase inoculum are transferred to the second inoculum fermentor. The medium of this second inoculum fermentation is similar to that of the first.

This second phase inoculum fermentation is also run ca. one day hours under controlled (pH[6]/T) conditions. After growth, this second phase inoculum is used to start the main fermentation.

Main fermentation:

Biosynthesis of endo-protease occurs during the main fermentation. To produce the enzyme of interest, a submerged, aerobic fed batch fermentation process is employed, using a stirred tank fermentor. This fermentor is equipped with devices for pH, temperature, oxygen and antifoam control, a top-mounted mechanical agitator and a bottom air sparger.

The culture medium used has been developed for both optimum growth of *Aspergillus niger* and optimum production of endo-protease. Before transfer of the inoculum, the empty fermentor is steam sterilized. Directly after inoculation until a few hours before the end of fermentation the fermentor is fed with aseptically introduced sterilized fermentation medium, with a similar composition as the seed fermentation with some additional mineral salts and vitamins added.

After a batch phase, the fermentor is continuously fed by aseptically introducing sterilized medium (containing equivalent raw materials as the batch medium) according to a preset feeding program.

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[5] This consists for example of glucose (as C-source), yeast extract (as N-source) and food grade antifoam.

[6] Organic salts and titrating agents like phosphoric acid and ammonia are used for pH control

Growth of the production organism and increase of enzyme production are regularly monitored in the main fermentation by analysis of aseptically collected samples.

Downstream processing

The downstream processing consists of the following steps:

- Killing the production strain
- Removal of the cell material
- Ultrafiltration
- Stabilization/Storage

Killing the production strain

After the fermentation is stopped, the temperature of the broth is decreased to 30° C. The production organism is killed by sodium benzoate at pH 4.0 ± 0.1 (incubation time 6 hours). The killing is validated by a microbial count after 0, 2, 4 and 6 hours.

Removal of the cell material

The temperature of the broth is decreased to 5 – 10°C. As endo-protease is excreted by the production organism into the medium, separation of cell material and product is a simple filtration process: the fermentation broth is filtrated using a membrane filter press, equipped with polyamide textile cloth, a flocculant and a filter-aid. This enables to eliminate the endo-protease from the cell material.

Ultrafiltration

The final step in the recovery is an ultrafiltration to concentrate the product. During this ultrafiltration, molecules with a molecular weight smaller than 5 –10 kD (depending on the shape of the molecules) and lower are removed from the broth.

The duration of the ultrafiltration is approximately 32 hours per fraction and is depending on the viscosity and filterability of the liquid (which varies due to natural variations in the raw materials).

During this process step the volume is reduced about 7 times, reducing the amount of low molecular weight molecules about these 7 times. The high molecule weight fraction of the proteins is unable to pass the membrane and so concentrated, whilst the concentration of the low molecule weight fraction remains the same.

The UF concentrate is subsequently germ filtrated.

Stabilization/Storage

This intermediate is stored at or below 5°C until further processing.

Stabilization and formulation

In order to obtain an end product, the UF concentrate is formulated with glycerol after which more glycerol is used to bring the product to the desired endo-protease activity of 5.5 PPU/ml.

Total Organic Solids (TOS)

The Total Organic Solids of the enzyme preparation were calculated from non-stabilized and un-standardized samples of 3 different fermentation batches, sampled before the required second UF concentration (see Section II.A.4).

Calculation of the TOS					
Batch number	Water (%)	Ash (%)	TOS (%)	Activity (PPU / g)	PPU / mg TOS
1	74.1	0.7	25.2	11.0	0.044
2	83.6	0.2	16.2	9.0	0.056
3	89.4	0.3	10.3	5.3	0.051
MEAN					0.050

HYGIENE

Good Food Manufacturing Practice

For optimal enzyme production, it is very important that hygienic conditions during the whole fermentation process are strictly controlled. Microbial contamination would immediately result in less growth of the production organism and consequently in a low yield of the wanted enzyme(s).

In addition to the microbial hygiene, it is also important that the raw materials and processing aids used during fermentation are of sound quality and do not contain contaminants such as pesticides or a high amount of heavy metals, which might affect the optimal growth of the production organism and thus enzyme yield.

Of course, the quality of the stock culture and the strict control of parameters as pH, temperature and aeration during fermentation are also of the utmost importance for optimal enzyme production and yield.

Thus, the commercial self-interest of any enzyme producer demands a strictly controlled fermentation process.

Enzyme fermentation experience since 1922 in the DSM (formerly Gist-brocades) factory at Seclin, France, has resulted in a solidly established Good Food Manufacturing Practice within the framework of a certified ISO system.

Technical measures:

The batches of **primary seed material**, also called Working Cell Bank (WCB), are always prepared from the so-called Master Cell Bank (MCB) in Laminar Air-flow (downflow) safety cabinets to ensure the absence of contamination. The batches are divided into a large number of vials for use in production over a long period of years

without any changes in strain- and production properties. In theory, a batch is large enough to last for about 10 years, depending on the strain viability and the fermentation frequency and thus the market demand.

The above procedures for preparation, preservation and storage are chosen to avoid degeneration and to secure genetic stability. All vials are clearly labelled and in revival of the culture, strict aseptic techniques are applied.

The **raw materials** used to make up the nutrient medium for the fermentation are added to mixing tanks and sterilised. The heat-treated nutrient solutions are then cooled for optimum cell growth and subsequently transferred to the fermentor.

The **fermentor** is a closed system. Air introduced into the fermentor is sterilised with a filter. Proper temperature conditions are maintained with cooling coils inside the fermentor.

Prior to inoculation, the fermentor is cleaned with solutions of food grade detergents, rinsed with water and then sterilised (empty) with steam.

All materials are pumped into the fermentor under overpressure via fixed connections which are equipped with self-closing valves. In this way, the sterilised nutrient medium from the mixing tank and the complete biomass broth from the inoculum fermentation are transferred aseptically to the main fermentor.

Microbial contamination during **fermentation** is prevented by the use of a large inoculum, carefully chosen optimum growth conditions for the production organism, overpressure in the fermentation vessel, and the use of sterile air.

The germ filtration during **downstream processing** additionally ensures that the end product is free of microbial contamination.

Control measures:

A new WCB is prepared from the MCB as soon as the previous batch becomes depleted or the concentration of viable cells decreases.

After preparation of a new WCB, samples are checked for identity, viability and microbial purity, using different temperatures (25, 30 and 37°C) and media, by enrichment and viewing morphology (colony shape and microscopy). If all these parameters are correct, the strain is tested for production capacity, first on laboratory scale and later on large scale production level. Only if the productivity and the product quality meet the required standards, the new WCB is accepted for further production runs.

The viability of the WCB is checked at least once a year.

The **raw materials** used in the fermentation process are checked to be of suitable purity and free of harmful substances. The ingredients used are tightly controlled to minimize the risk of contaminants that would inhibit growth of the production organism or enzyme production.

The Quality Control (QC) Department provides assurance that these materials comply with appropriate specifications.

During the **seed fermentation** manual samples are taken aseptically from a sampling port on the fermentor for analysis in the laboratory. Samples are checked for pH and microbiological quality.

During the **main fermentation** the correct temperature, pH and dissolved oxygen content are monitored and automatically adjusted throughout the process to ensure optimal enzyme production and a consistent process and thus product.

During the main fermentation manual samples are taken aseptically from a sampling port on the fermentor for analysis in the laboratory. These samples are analysed for such parameters as microbial purity, pH, viscosity and enzyme activity. If microbial controls show that contamination has occurred, the fermentation will be discontinued.

Also during **downstream processing,** most particularly at the end of the UF concentration, samples are taken and checked for activity, dry matter, pH, specific gravity and the level of microbial contamination.

After **stabilisation**, the semi-finished product is analysed for colour, endo-protease activity, dry matter content, and particle size and checked for microbiological contamination. Only if the product meets the in-process specifications, it will be accepted as a basis to formulate the final commercial product.

5. Specification for identity and purity

The final enzyme preparations are analysed for the following parameters:

Parameter	**Norm**
Endo-protease activity	5.0 – 5.8 PPU/g
Appearance	Light brown to brown liquid
pH	3.8 – 4.2

In addition, according to the general specifications for enzyme preparations used in food processing as established by the Joint Expert Committee of Food Additives of the FAO/WHO (JECFA, 2006), The Food Chemical Codex 7th edition and according to the French legislation (Arrêté, 2006), the endo-protease preparations from *Aspergillus niger* fulfil the following demands:

Parameter	**Norm**
Lead	≤ 5 mg/kg
Cadmium	≤ 0.5 mg/kg
Mercury	≤ 0.5 mg/kg
Arsenic	≤ 3 mg/kg
Standard plate count	≤ 5x10^4/g
Coliforms	≤ 30/g
Salmonella	0/25 g
Escherichia coli	0/25 g
Anaerobe sulphite reducing	< 30/g
Staphylococcus aureus	0/g
Antimicrobial activity	Absent by test
Mycotoxins	Absent by test

Total Microbial Count

As is explained above and proven below, the endo-protease preparation complies with international purity standards and addition to foodstuff (in an amount of maximally 1% on basis of w/w) will therefore not cause an increase in the total microbial count.

Heavy metals

As can be seen in the 3 Certificates of Analysis given in Annex II.A.5-1, the endo-protease preparations comply with the specifications for heavy metals as recommended by JECFA.

Microbiological contaminants

As can be seen in the 3 Certificates of Analysis given in Annex II.A.5-1, the endo-protease preparations comply with the specifications for microbial contaminants as recommended by JECFA.

Test for absence of the production strain

For proprietary reasons, it is very important for each enzyme producer that the final commercial product does not contain viable production organisms. In the case of endo-protease production, the cells are killed off at the end of the fermentation (see Section II.A.4), which ensures that the final product is free from the production organism *Aspergillus niger.*

Test for absence of antibiotic activity

As can be seen in the 3 Certificates of Analysis given in Annex II.A.5-1, the endo-protease preparations do not contain antibiotic activity.

Test for absence of toxins

Although absence of mycotoxins was mentioned in the specification requirements for fungal enzymes as laid down by the Food Chemicals Codex ("FCC") and JECFA in the past, this requirement has recently been deleted.

Instead, the FCC (7th edition) mentions the following: "*Although limits have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants."*

In the General Specifications for enzyme preparations laid down by JECFA in 2006, the following is said: *"Although nonpathogenic and nontoxigenic microorganisms are normally used in the production of enzymes used in food processing, several fungal species traditionally used as sources of enzymes are known to include strains capable of producing low levels of certain mycotoxins under fermentation conditions conducive to mycotoxin synthesis. Enzyme preparations derived from such fungal species should not contain toxicologically significant levels of mycotoxins that could be produced by these species."*

Instead of performing analyses on the endo-proteinase preparations, DSM therefore decided it more appropriate to test whether the endo-proteinase-producing microorganism possesses the capacity to produce mycotoxins under the conditions of the endo-proteinase production process. The test showed that the production strain does not produce any known toxins under these conditions (see Annex II.A.5-2). This means that further testing of endo-proteinase preparations is superfluous.

Known allergens

The formulation the endo-protease is sold in does not contain allergens.

Presence of residues in the final product

In the classical beer manufacturing process, one of the last steps is the pasteurization of the beverage. As mentioned earlier, our enzyme is heat-labile and as a result is inactivated in the pasteurization step. With the analytical method given in this dossier, which is 1 to 2 orders of magnitude more sensitive than the FCC HUD method, no remaining activity of the enzyme can be determined in the final product.

C. Information related to the safety of an enzyme processing aid

1. General information on the use of the enzyme as a food processing aid in other countries

Applications for the use of endo-protease from *Aspergillus niger* have already been filed successfully in France, Russia, Denmark and China. In the USA protease from *Aspergillus niger* has been notified as GRAS (GRN 000089).

2. Information on the toxicity of the enzyme processing aid

Enzymes produced by *Aspergillus niger* have already been used for food production since several decades. Various enzymes from *Aspergillus niger* have been evaluated for their safety by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO (WHO Food Additives Series 6 (1975) and 22 (1988). All these enzymes have received an ADI "not specified" by JECFA, meaning that the amount that can be added to food is determined only by Good Manufacturing Practice.

The Australian New Zealand Food Standards code contains a long list of enzymes produced by *Aspergillus niger* including catalase, amylase, pectinesterase, glucose oxidase and asparaginase. Recently, lipase produced by a strain from the same strain lineage was approved.

In the USA, FDA has previously affirmed as GRAS several enzyme preparations from *Aspergillus niger* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *Aspergillus niger* strains, such as carbohydrases, proteases, pectinases, glucose oxidase and catalase (GRN 000089), lactase (GRN 000132), and lipase (GRN 000111 and GRN 000158). The FDA recently summarized the safety of microorganisms, including *Aspergillus niger*, used as a host for enzyme-encoding genes (Olempska-Beer et al., 2006). Even more recently, a phospholipase A2 (GRN 000183), an asparaginase (GRN 000214) and a lipase (GRN 000296) preparation from a genetically modified *Aspergillus niger* strain, derived from the same strain-lineage as the *Aspergillus niger* strain described in this dossier, have been notified as GRAS.

Endo-proteases are ubiquitous in nature and can be found in eukaryotes, bacteria and archae (Venäläinen *et al*., 2004). Already in 1958 Underkofler *et al.* described the use of endopeptidases in food production. In addition, the protease activity was included in the safety evaluation by Pariza and Johnson (2001), several proteases being enzyme activities used in the food processing industry.

Endo-protease[7] is an enzyme that is already approved and is used for many years in food applications (Gass & Khosla 2007).

[7] Protease is also named proteinase and peptidase.

In Australia/New Zealand, proteinases from several sources have been approved including *Aspergillus niger*, *Aspergillus oryzae*, *Rhizomucor* m*iehei* as well as several *Bacillus* species (for more details See section I.2).

A peptidase enzyme preparation form *Aspergillus niger* has been notified as Generally recognized as safe (GRN000089). Several other peptidases were notified as GRAS: peptidase from *Aspergillus oryzae* (GRN000090) and chymosin from *Trichoderma reesei* (GRN000230).

In addition to the above, the safety of the current endo-protease of *Aspergillus niger* can be discussed in the light of substantial equivalence.

Several expert groups, as well as FDA and FDA scientists, have discussed the concept of substantial equivalence relative to food safety assessment. Essentially, all these groups conclude that if a food ingredient is substantially equivalent to an existing food ingredient known to be safe, then no further safety considerations other than those for the existing ingredient are necessary.

In addition, FDA has applied this concept in the determination that several enzyme preparations are safe for use in food. In particular, FDA has considered differences in glycosylation between enzyme proteins. FDA has also stated that enzyme proteins demonstrated to be substantially equivalent to enzymes known to be safely consumed but having differences in specific properties due to chemical modifications, or site-direct mutagenesis, would not raise safety concerns.

There are no agreed-upon criteria by which substantial equivalence is determined. Considering enzymes produced by micro-organisms the enzyme activity and intended use, the production organism and the process conditions should be taken into account.

As described in Sections D and E the endo-protease described in this dossier is produced by an *Aspergillus niger* strain derived from the so called GAM/ISO lineage. Strains from this lineage have already proven to be safe hosts for the over expression of enzymes in general, as shown by the safety studies performed on other enzyme production strains derived from this lineage (Van Dijck et al., 2003). The production process, the production strain, the construction of the production strain and the raw materials are standardized. The current endo-proteinase producing strain was build and produced according to this concept.

So it can be concluded that the resulting enzyme product containing endo-protease activity is substantial equivalent concerning enzymatic activity and use, production strain and production process.

Based on the above information, DSM believes that the endo-protease enzyme and its producing organism *Aspergillus niger* are safe for its intended use.

To confirm the assumption that endo-protease from *Aspergillus niger* administered via the oral route would not have any toxic properties and to further establish the toxicological safety of the use of endo-proteinase from *Aspergillus niger* in food, the following studies were performed:

- Subacute (14-day) oral toxicity study (range-finding)
- Subchronic (90-day) oral toxicity study

- Ames test
- Chromosomal aberration test, *in vitro*

All tox-studies have been performed with the same batch (batch JLL 03 006 IDF, further called 'tox-batch') which was produced according to the normal commercial production process. It has an activity of 11.0 PPU/g and a TOS content of 25.2%.

All safety studies, except the 14-day dose range-finding study, were performed according to internationally accepted guidelines (OECD) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

Summarizing the results obtained from the several toxicity studies performed as given in the following Sections, the following conclusions can be drawn:

- The tox-batch did not show any mutagenic or clastogenic activity under the given test conditions
- Based on the results of the sub-chronic oral toxicity study the No Observed Adverse Effect Level (NOAEL) of the tox-batch is approximately 20 g product/kg body weight/day (i.e. 5040 mg TOS/kg body weight/day) (highest dose level tested).
- In conclusion, the overall NOAEL of the tox-batch is 2135 mg/kg body weight/day, which corresponds with 1008 mg TOS or 220 PPU/kg body weight/day.

Based on the NOAEL and the amounts of inactive enzyme present in the final food, the Margin of Safety (MoS) for human consumption was calculated for the estimation of the daily consumption of the enzyme:

Estimated Daily Intake (EDI) in the Netherlands

The estimated daily intake in the Netherlands is given in section II.F.4.

Based on the data given in Sections II.F.2 and II.F.4 and on a body weight for a person of 60 kg, the following calculation can be made:

Residual amount of (denatured) enzyme in final food (PPU/l beer)	**Residual amount of (denatured) enzyme in final food (mg TOS/l beer)**	**90th percentile intake level (l food/ per person/day)**	**Estimated daily intake of (denatured) enzyme (mg TOS/kg bw/day)**
0.75	15	5	1.25

The Margin of Safety (MoS) can be calculated by dividing the NOAEL by the Estimated Daily Intake. With an overall NOAEL of 2135 PPU/kg body weight/day the following MoS can be calculated:

MoS via EDI: 5040/1.25 = 4032

Regarding the height of the MoS, it was concluded that further testing of the safety of the product is not meaningful.

14-days oral toxicity

The study procedures were based upon the following guidelines:

- EEC Directive 96/54/EEC, B7 Repeated Dose (28 days) toxicity (oral), 1996
- OECD 407, Repeated Dose 28-day Oral Toxicity Study in Rodents, 1995

The subacute oral toxicity was examined in a 14-day study with four groups of five male and five female Wistar rats, which received the tox-batch (batch JLL 03 006 IDF), by oral gavage, at dose levels of 2000, 7000 and 20000 mg/kg bw/day, equivalent to 518, 1813 and 5180 mg dry matter/kg bw/day. A similarly constituted vehicle control group received double distilled water.
The following parameters were evaluated:
Clinical signs daily; body weights and food consumption weekly; clinical pathology, macroscopy and organs weights at termination.

No histopathological examination of organs and tissues was performed.

Results

2000 mg/kg/day	No treatment-related findings noted
7000 mg/kg/day	No treatment-related findings noted
20000 mg/kg/day	No treatment-related findings noted

Conclusion

From the results a No Observed Effect Level (NOEL) of 20000 mg/kg/day was concluded.
Dose levels for the 90-day oral gavage study in Wistar rats were selected to be 0, 2000, 7000 and 20000 mg/kg bw/day (see Annex II.C.2-1).

90-days oral toxicity

The study was based on the following guidelines:

- EEC Directive 87/302/EEC, B Sub-chronic Oral Toxicity test: 90 day repeated oral dose using rodent species, L 133, May 1988.
- OECD 408, Repeated Dose 90-day Oral Toxicity Study in Rodents, 1998.
- US-EPA Pesticides and Toxic Substances (71010 EPA 712-C-96-199) “health effects Test Guidelines” OPPTS 870.3100 90-day Oral Toxicity Public Draft June 1996.

The sub-chronic oral toxicity of the tox-batch (batch JLL 03 006 IDF) was examined in a 90-day study with groups of 10 male and 10 female Wistar rats. The rats received the tox-batch daily by gavage at dose levels of 2000, 7000 and 20000 mg/kg bw/day, equivalent to 518, 1813 and 5180 mg dry matter/kg bw/day. A similarly constituted vehicle control group received double distilled water.

The following parameters were evaluated:

Clinical signs, functional observations, body weight, food consumption and ophthalmoscopy. At termination: clinical pathology, macroscopy, organ weights and microscopy.

Results

2000 mg/kg/day	No treatment-related findings
7000 mg/kg/day	No treatment-related findings
20000 mg/kg/day	Increased body weights in females and reduced food consumption in males, in comparison with the vehicle controls

Conclusion

Based on the absence of any corroborative functional disturbances and morphological changes in the high-dose animals, the alterations in body weights and food consumption were considered to be attributed to the extra energy intake through the enzyme preparation derived from *Aspergillus niger* (GEP 44) and of no toxicological significance. Therefore, a No Observed Adverse Effect Level (NOAEL) of 20000 mg/kg bw/day was concluded (see Annex II.C.2-2).

Mutagenicity tests

AMES test (see Annex II.C.2-3)

The study procedures were based upon the following guidelines:

- OECD 471 Genetic Toxicology: Bacterial Reverse Mutation Test (adopted July, 1997)
- EEC Directive 92/69/EEC, EEC publication No L383; B13 Mutagenicity: Escherichia coli Reverse mutation Assay with Wp2uvrA only. B14 Mutagenicity: Salmonella Typhimurium – Reverse Mutation Assay (adopted Dec., 1992)

The tox-batch (batch JLL 03 006 IDF) was examined for its mutagenic potency in four histidine-requiring Salmonella typhimurium mutant strains: TA 98, TA 100, TA 1535 and TA 1537 and one tryptophan-requiring Escherichia coli mutant strain WP2uvrA in two independent experiments.

Tester bacteria were exposed to five concentrations ranging from 62 to 5000 µg dry matter/plate in the absence and presence of a rat liver-derived metabolic activation system (S-9 mix).

Negative (water) and positive controls were run simultaneously with the test.

In the first assay an increase in the number of reverse mutation together with a (slightly) more dense the bacterial background lawn was observed, indicating the presence of histidine or proteins. Therefore the second assay was performed according to the treat-and-plate method to exclude false positive results. In the second assay no increase in the number of revertants was observed. Precipitation and toxicity was not observed.

The positive control substances, sodium azide, daunomycine, methylmethane-sulphonate, 4-nitroquinolone, 2-aminoanthracene, and 9-aminoacridine gave the expected increase in the number of revertants.

Based on the results of this study it is concluded that the tox-batch is not mutagenic in the Salmonella typhimurium reverse mutation assay and the Escherichia coli reverse mutation assay.

Chromosomal aberration test (see Annex II.C.2-4)

The study procedures were based upon the following guidelines:

- OECD 473 Genetic Toxicology: In vitro Mammalian Chromosome Aberration Test (adopted July, 1997)

The tox-batch (batch JLL 03 006 IDF) was examined for its effect on the induction of chromosomal aberrations in cultured human peripheral lymphocytes in the presence and absence of a metabolic activation system (Acroclor-1254 induced rat liver S9-mix).

Negative (culture medium) and positive controls were run simultaneously with the test substance.

In the absence of S9-mix the tox-batch was tested up to 5000 µg/ml for a 4 h and 24h treatment time with a 24 h fixation time in the first experiment. In the second experiment the tox-batch was tested up to 5000 µg/ml for a 24 h treatment time with a 24 h fixation time as well as for a 48 hour treatment time with a 48 h fixation time.
In the presence of 1.8% (v/v) S9-fraction, the tox-batch was tested up to 5000 µg/ml for a 4 hour treatment time with a 24 h fixation time in the first experiment and for a 4 h treatment time with a 48 h fixation time in the second experiment.

Positive control chemicals, mitomycin C and cyclophosphamide, both produced a statistically significant increase in the incidence of cells with chromosome aberrations, indicating that the test conditions were adequate and that the metabolic activation system (S-9) functioned properly.

In the two experiments, in both the absence and presence of S9-mix, the tox-batch did not induce a statistically significant increase in the number of cells with chromosome aberrations at any of the dose levels at any time point analysed.

It is concluded that the tox-batch is not clastogenic to human lymphocytes under the experimental conditions of this in vitro test.

D Additional information related to the safety of an enzyme processing aid derived from a micro-organism

1. Information on the source micro-organism

Identification and Taxonomy

Strain GAM53 (DS3045) – which is the parent of the host strain ISO-508 (DS 38556) – as well as many of the derived recombinant production strains have been taxonomically identified by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS). This is an independent, internationally recognised laboratory.

As can be seen in Annexes II.D.1-1 and II.D.1-2, respectively, both the parental strain GAM53 (DS 3045) and the recombinant production strain GEP-44 were determined by the usual classical identification as *Aspergillus niger* according to the description of v.Tieghem.

The species *Aspergillus niger* has been subject to several taxonomic treatments. Based on molecular biological techniques, the current concept is that names such as *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus usamii*, *Aspergillus satoi* and *Aspergillus tubingensis* are belonging to the aggregate *Aspergillus niger van Tieghem* (Kusters-van Someren et al., 1991).

A. niger is classified as follows:

Kingdom	:	FUNGI
Division	:	EUMYCOTA
Subdivision	:	DEUTEROMYCOTINA
Class	:	HYPHOMYCETES
Order	:	Moniliales
Family	:	Moniliaceae
Genus	:	*Aspergillus*
Sub-genus	:	Circumdati
Section	:	Nigri (= *Aspergillus niger* group)
Species	:	*Aspergillus niger*

Origin of the strain

The recipient organism used in the construction of the endo-protease production strain is a glucoamylase (also called amyloglucosidase) and protease negative *Aspergillus niger* strain designated ISO-508 and stored in the DSM Culture Collection as DS 38556.

The strain ISO-508 was derived from the fully characterized DSM *Aspergillus niger* strain GAM-53 (DS 3043) by genetic modification. The strain GAM-53 was derived by several classical mutagenesis steps from *Aspergillus niger* strain NRRL 3122, a strain purchased from the Culture Collection Unit of the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois, USA.

The fully characterized strain *Aspergillus niger* GAM-53 was isolated by DSM (then: Gist-brocades) in 1982 and selected for its enhanced production of the endogenous enzyme glucoamylase. Since that time, strains of the GAM-lineage have been used at DSM for the large-scale production of glucoamylase, an enzyme that is utilized worldwide in the starch processing industry.

The strain GAM-53 is being used to construct a new generation of strains according to the 'design and build' concept, in which introduced genes are targeted ('plugged') to a predetermined region of the genome. The exact technique used to construct such 'plug bugs' (designated as 'ISO-strains') and its advantages are described in literature (Selten *et al*., 1995 and van Dijck *et al*., 2003). ISO-strains were used for the construction of a variety for production strains such as arabinofuranosidase (donor: *Aspergillus niger*), phytase (donor: *Aspergillus niger*), pectin methyl esterase (donor: *Aspergillus niger*), phospholipase A2 (donor: porcine pancreas), glucoamylase (donor: *Aspergillus niger*), xylanase (donor: *Aspergillus niger*), endo-polygalacturonase (donor: *Aspergillus niger*), proline specific endo-protease (donor: *Aspergillus niger*), and amylase (donor: *Aspergillus niger*), asparaginase (donor: *Aspergillus niger*) and a lipase.

The recipient organism ISO-508 used in the construction of the endo-protease product strain was derived from GAM-53 as follows:

The strain GAM-53 contains 7 loci (i.e. the promoter and coding sequences) for the glucoamylase gene. These 7 loci were removed, creating so-called 'plug-sites' (also called Δ*gla*A loci) into which expression units containing various genes can be integrated ('plugged'). The 7 'plug-sites' were each provided with unique restriction sites (also called 'DNA-flags'), marking the location of the 'plug-sites' on the genome. A schematic representation of how these plug-sites can be detected analytically is given in Annex II.D.1-3.

In addition, the gene coding for the major protease (*pep*A) was inactivated by established rDNA techniques and the strain's capacity to secrete proteins was improved by classical mutation and selection.

The resulting 'plug bug', ISO-508, was classified as a self cloned GMO by the Dutch competent authorities (see Annex II.D.1-4) and was used to construct the endo-protease production strain GEP-44.

2. Information on the pathogenicity and toxicity of the source micro-organism

Aspergillus niger is known to naturally occur in foods. The fungus is commonly present in products like rice, seeds, nuts, olives and dried fruits.

For several decades, *Aspergillus niger* has been safely used in the commercial production of organic acids and various food enzymes, such as glucose oxidase, pectinase, alpha-amylase and glucoamylase. Industrial production of citric acid by *Aspergillus niger* has taken place since 1919.

This long experience of industrial use has resulted in a good knowledge of the characteristics of *Aspergillus niger* and understanding of the metabolic reactions.

The long industrial use and wide distribution of *Aspergillus niger* in nature has never led to any pathogenic symptoms. The non-pathogenic nature has been confirmed by several experimental studies. *Aspergillus niger* is therefore generally accepted as a non-pathogenic organism (Schuster *et al*., 2002).

Even though products from *Aspergillus niger* have been used in food for many decades, there has never been any evidence that the industrial strains used are able to produce toxins. The non-toxicogenicity has been confirmed by toxicological tests, as well as batch testing of the various end products for toxins.

The toxicological studies performed on various enzyme preparations from *Aspergillus niger* provided the basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO in 1987 (JECFA, 1988). Although not justified by the results of the toxicological studies, JECFA first allocated a numerical Acceptable Daily Intake (ADI) to enzyme preparations of *Aspergillus niger*, based on the concern that some strains may produce unknown toxins. Two expert reports submitted to JECFA in 1988 concluded that the production of toxins was highly unlikely (see Annex II.D.2-1). The long history of use as an enzyme source, the numerous toxicological studies and the two expert reports caused JECFA to review its decision in 1990 and change the ADI for enzyme preparations derived from *Aspergillus niger* into "not specified" (JECFA,1990).

In addition to the positive evaluation of JECFA, countries, which regulate the use of enzymes, such as the USA, France, Denmark, Australia and Canada, have accepted the use of enzymes from *Aspergillus niger* in a number of food applications.

Strains belonging to the *Aspergillus niger* GAM-lineage as well as the host (recipient) strain ISO-508 from DSM were declared suitable host strains for the construction of genetically modified organisms belonging to Group I safe microorganisms by the Dutch authorities.

The *Aspergillus niger* GAM-53 strain, which is being used as the parental strain of the host organism, has already been used as host for the selection of genetically modified production strains, by the process of random integration, for the production of the enzymes phytase and xylanase. DSM uses these strains on industrial scale since 1991 and 1996, respectively. The first production strains, based on targeted integration of genes of interest in ISO hosts, are in use on large scale since 1998.

The recombinant endo-protease production strain GEP-44C (DS49221) has been classified by the Dutch authorities as well as the French Genetic Committee as a Group I safe micro-organism. Consequently, the strain was approved for large-scale production of endo-protease in the DSM factory in Seclin, France.

Based on the genetic modification performed (see Section E), there are no reasons to assume that the recombinant production strain should be less safe than the original GAM-53 (DS 03043) strain. In fact, it has been shown that the DSM GAM/ISO lineage of *Aspergillus niger* strains are safe hosts for the over-expression of enzymes to the extent that for new enzymes the safety is already covered by the safety studies performed on other enzyme production strains derived from this lineage and consequently new safety studies are superfluous (Van Dijck *et al*., 2003).

At the end of the fermentation, the recombinant production organism is effectively killed off.

Specific tests have been performed to confirm that the recombinant endo-protease production strain is not able to produce any toxins under conditions, known to induce toxin production, nor under standard endo-protease production conditions. The results of these tests showed that the production strain does not produce any known toxins under these conditions (see Annex II.A.5-2).

3. Information on the genetic stability of the source organism

The strains belonging to the *Aspergillus niger* GAM-lineage - from which both the host ISO-508 and the present recombinant endo-proteinase production strain GEP-44 are derived - are genetically stable strains. The whole GAM-lineage is stored for more than 30 years at the DSM laboratory. New cultures are frequently derived from stock material and tested after many generations on morphological-, growth-, production- and product characteristics. These characteristics remain stable except that after plating out a low frequency of morphologic dissimilar colonies are found. This, however, is a normal phenomenon observed for the parental as well as the highly selected industrial strains. The stability of production strains from this ISO strain lineage, such as the endo-protease production strain, in terms of behaviour in strain management and in terms of enzyme production characteristics, does not differ from the parental GAM-strains or from production strains constructed by random integration.

Since the endo-protease expression unit is integrated into the genome and since the expression unit does not contain an *Escherichia coli* origin for replication (all *Escherichia coli* sequences have been removed from the plasmid prior to transformation), it is not possible that the expression unit will be transferred from the *Aspergillus niger* production organism to another, non-related, organism.

E Additional information related to the safety of a processing aid derived from a genetically-modified micro-organism

1. Information on the methods used in the genetic modification of the source organism

In the genetic modification two expression cassettes are used: the endo-protease cassette and the selectable marker cassette. They are based on the endo-protease expression plasmid and the selectable marker plasmid respectively.

Endo-protease expression plasmid

The endo-protease expression plasmid pGBTOPGEP-1 contains strictly defined *Aspergillus niger* chromosomal elements (parts of the *Aspergillus niger* glucoamylase locus, which are the glucoamylase promoter and a sequence flanking the glucoamylase *glaA* gene at the 3' side), the endo-protease gene, and DNA from a well-characterized *Escherichia coli* vector pTZ18R. **These pTZ18R sequences are removed prior to transformation of the endo-proteinase expression cassette into the host.**

The map of plasmid pGBTOPGEP-1 is shown in Annex II.E.1-1.

Selectable marker plasmid

The selectable marker plasmid pGBAAS-1 contains the same defined parts of the *Aspergillus niger* glucoamylase locus as the expression plasmid, the *Aspergilus nidulans amdS* (acetamidase) selectable marker gene and DNA from a well-characterized *Escherichia coli* vector pTZ18R. **These sequences are removed prior to transformation of the *amdS* selection cassette into the host.**

The map of plasmid pGBAAS-1 is shown in Annex II.E.1-2.

Transformation and selection of the final production strain

The endo-protease expression cassettes and the selection marker cassettes, **both completely devoid of any *Escherichia coli* DNA sequences**, are integrated into the genome of the recipient organism ISO-508 by co-transformation following standard methodology. Due to the homology in the *gla*A promoter and 3'-*gla*A terminator parts of the two expression units, they are targeted to one of the seven ΔglaA loci.

Transformants are selected on their ability to utilize acetamide as sole carbon source. By further analysis transformants are selected that have multiple copies of the endo-protease expression cassette and one or more copies of the selection marker cassette integrated into one of the Δ*gla*A loci of the recipient strain. The selection of these transformants was done by PCR analyses, applying endo-protease and *gla*A specific primers.

By counter-selection on fluoro-acetamide containing plates, a natural variant of a transformant was selected in which the *amd*S selection marker was deleted as a result of a natural internal recombination event.

Schematically the transformation and the counter-selection is depicted in Annex II.E.1-3.

The absence of the *amd*S marker was confirmed by Southern analysis. The resulting organism is thus not only totally free of *Escherichia coli* DNA, but also of the *amd*S selection marker; see Annex II.E.1-4.

Starting with such an *Escherichia coli*- and *amd*S-free variant it is possible to multiply the region comprising the expression unit(s) and the "DNA-flag" into the other ∆*gla*A loci by so-called "gene conversion" (Selten *et al*., 1998), a natural spontaneous recombination event which does not involve mutagenic treatment. Strains that have an increase in the copy number of the "DNA flag" marking the filled ∆*gla*A locus and a consequent loss of the other "DNA-flags" can easily be identified by DNA gel electrophoresis (see also Annex II.E.1-5 and II.E.1-6).

From the available recombinants a strain was chosen that contained sufficient gene copies to allow for commercial attractive expression levels of the endo-proteinase enzyme: this strain was designated GEP-44.

F. Information related to the dietary exposure to the processing aid

1. A list of foods or food groups likely to contain the processing aid or its metabolites

DSM endo-protease is proposed for use in the preparation of beer.

In Annex II.F.1-1 a publication on the application of endo-protease in brewery is given. It confirms the benefits of using the endo-protease in beer manufacture.

2. The levels of residues of the processing aid or its metabolites for each food or food group

The enzyme aids the proteolysis of the barley or malted barley proteins during the beer production process, preferentially after the boiling step. Thereby it prevents the appearance of haze during (cold) storage due to polyphenol – protein interactions. As mentioned in Section II.A.3, the endo-protease is inactivated in the brewing process. As indicated in section II.A.5, no endo-protease activity could be determined in the beer.

Based on the information given in Section II.A.4, the following calculation can be made:

Final food	Enzyme use level in food ingredient	Amount of ingredient in final food	Residual amount of (inactivated) enzyme in final food	Amount of TOS in final food
Beer	0.75 PPU/l beer	100%	0.75 PPU/l beer	15 mg/l beer

Maximum amount of enzyme preparation to be used in each foodstuff

Enzyme preparations are generally used in *quantum satis*. The average dosage of the enzyme depends on the application, the type and quality of the raw materials used, and the process conditions.

The endo-protease preparation will be used at a maximum of 0.75 PPU/l beer.

Main reaction products and possible reaction products not considered normal constituents of the diet

Endo-proteases are ubiquitous in nature and can be found in eukaryotes, bacteria and archae (Venäläinen *et al*., 2004). Already in 1958 Underkofler *et al.* described the use of endopeptidases in food production.

The reaction products of the enzymatic conversion of proteins by the enzyme are peptides and amino acids.

During the fermentation of the beer (by yeast), or during malting/mashing, most of the (malt) proteins and peptides are subject to (enzymatic) degradation even without the additional effect of the endo-protease: This process occurs by native proteases

originated from the malting stage and/or the yeast. So, the hydrolysis of proteins and peptides as such is not a new phenomenon.

Since the enzyme is inactivated during the brewing process, no further reaction products will be formed after that. The inactivated enzyme remains inert in the food as any other protein.

Possible effects on nutrients

Based on the information given above and in section II.A.3, there is no basis to believe that there will be any effect on nutrients apart from intentional effects on the lipids.

3. The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid

The penetration percentage of Brewers Clarex is difficult to predict but from a company point of view we aim at a very high penetration percentage.

4. Information relating to the levels of residues in foods in other countries

Estimated Daily Intake (EDI) in the Netherlands

The EDI was calculated based on the maximal dose levels and consumption data in The Netherlands (Voedingscentrum, 1998). The mean consumption for the overall population in the study is 159 g alcoholic beverages/day with a 90th percentile of 318 g/day[8]. Based on the data given in Section II.F.2 and a body weight for a person of 60 kg, the following calculation can be made:

Residual amount of (denatured) enzyme in final food (PPU/l beer)	**Residual amount of (denatured) enzyme in final food (mg TOS/l beer)**	**90th percentile intake level (g food/ per person/day)**	**Estimated daily intake of (denatured) enzyme (PPU/kg bw/day)**
0.75	15	318	0.004

[8] 90th percentile is 2 times the intake level (CFSAN, 2006).

Estimated Daily Intake (EDI) in the USA

The Estimated Daily Intake was calculated based on the maximal dose levels and consumption data in The United States (Wilkinson-Enns et al. (1997)). The mean consumption for men 20 years and over (worst case category) in the study is 216 g beer and ale/day with a 90th percentile of 432 g/day[8]. Based on the data given in Section II.F.2 and a body weight for a person of 60 kg, the following calculation can be made:

Residual amount of (denatured) enzyme in final food (PPU/l beer)	**Residual amount of (denatured) enzyme in final food (mg TOS/l beer)**	**90th percentile intake level (g food/ per person/day)**	**Estimated daily intake of (denatured) enzyme (PPU/kg bw/day)**
0.75	15	432	0.005

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H. Literature

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III APPLICATION CHECKLIST

General Requirements (3.1)	
■ Form of application ■ *Executive Summary* ■ *Relevant sections of part 3 identified* ■ *Pages sequentially numbered* ■ *Hard copies capable of being laid flat* ■ *Electronic and hard copies identical* ■ Applicant details ■ Purpose of the application	■ Justification for the application ■ Information to support the application ■ Assessment procedure ■ Confidential Commercial Information □ *Confidential material separated in both electronic and hard copy* ■ Exclusive Capturable Commercial Benefit ■ International standards ■ Statutory Declaration
Food Additives (3.3.1)	
Not applicable	
Processing Aids (3.3.2)	
■ Type of processing aid ■ Identification information ■ Chemical and physical properties ■ Manufacturing process ■ Specification information □ Industrial use information (chemical only) □ Information on use in other countries (chemical only) □ Toxicokinetics and metabolism information(chemical only) □ Toxicity information (chemical only) □ Safety assessments from international agencies (chemical only)	■ Information on enzyme use on other countries (enzyme only) ■ Toxicity information of enzyme (enzyme only) ■ Information on source organism (enzyme from micro-organism only) ■ Pathogenicity and toxicity of source microorganism (enzyme from micro-organism only) ■ Genetic stability of source organism (enzyme from micro-organism only) ■ Nature of genetic modification (PA from GM micro-organism only) ■ List of foods likely to contain the processing aid ■ Anticipated residue levels in foods ■ Percentage of food group to use processing aid ■ Information on residues in foods in other countries(if available)
Nutritive Substances (3.3.3)	
Not applicable	