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## **Supporting document 1**

Risk and Technical Assessment – Application A1241

Pectinesterase from GM *Aspergillus oryzae* as a processing aid

### **Executive summary**

AB Enzymes applied to Food Standards Australia New Zealand (FSANZ) to amend Schedule 18 – Processing Aids of the Australia New Zealand Food Standards Code (the Code) to include pectinesterase (EC 3.1.1.11), a pectinase, as a processing aid. It is produced from a genetically modified (GM) strain of *Aspergillus oryzae* (*A. oryzae*) containing the pectinesterase gene from *Aspergillus tubingensis* (*A. tubingensis*). The specific name for the production organism is *A. oryzae* strain AR-962. The proposed use of pectinesterase is as a processing aid in the manufacture and/or processing of fruit and vegetable juices/products; and in the production of coffee, flavouring substances and wine.

FSANZ has undertaken an assessment to determine whether the enzyme achieves its technological purpose in the quantity and form proposed to be used and to evaluate public health and safety concerns that may arise from the use of this enzyme.

FSANZ concludes that the proposed use of pectinesterase as an enzyme processing aid in the manufacture and/or processing of fruit and vegetable juices/products; and in the production of coffee, flavouring substances and wine is consistent with its typical function of hydrolysing pectin in those foods. Analysis of the evidence provides adequate assurance that the use of the enzyme, in the form and requested amount, is technologically justified and has been demonstrated to be effective in achieving its stated purpose.

Pectinesterase performs its technological purpose during the production of foods by breaking down plant cell walls, and is not performing a technological purpose in the final food, therefore functioning as a processing aid as defined in the Code. There are relevant identity and purity specifications for the enzyme in the Code.

No public health and safety concerns were identified in the assessment of pectinesterase from a modified strain of *A. oryzae* under the proposed conditions of use.

The host strain is neither pathogenic nor toxigenic. No food safety hazard was identified in the isolation and use of the pectinesterase gene from *A. tubingensis*. Analysis of the production strain (*A. oryzae* AR-962) confirmed the presence and stability of the introduced DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro*. The no observed adverse effect level (NOAEL) in a 90-day oral gavage study in rats was 1000 mg TOS<sup>1</sup>/kg bw/day, which was the highest dose tested. The theoretical maximum daily intake (TMDI) was calculated by FSANZ to be 0.715 mg TOS/kg bw/day. Comparison of the NOAEL and the TMDI gives a margin of exposure (MOE) of approximately 1400.

Bioinformatic analysis indicated that the enzyme showed no significant homology with any known toxins or venoms. A degree of homology between the enzyme and two pollen allergens was found. Taking into account that neither of these allergens is a food allergen and that only low levels of the enzyme processing aid are expected to be present in final food products, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

Based on the reviewed data, it is concluded that, in the absence of any identifiable hazard, an Acceptable Daily Intake (ADI) 'not specified' is appropriate.

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<sup>1</sup> TOS: Total organic solids.

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# 1 Introduction

The applicant, AB Enzymes, is seeking permission for pectinesterase (EC 3.1.1.11) for use as a processing aid. The enzyme is produced from a genetically modified (GM) strain of *Aspergillus oryzae* (*A. oryzae*) containing the pectinesterase gene from *Aspergillus tubingensis* (*A. tubingensis*). The specific name for the production strain is *A. oryzae* strain AR-962.

Pectinesterase is a pectinase suitable for hydrolysing pectin and can be used in the manufacture and/or processing of fruit and vegetable juices/products; and in the production of coffee, flavouring substances and wine.

Marketed via an enzyme preparation, if approved, the enzyme is to be used in combination with polygalacturonase being assessed under FSANZ Application A1240 (A1240 was submitted simultaneously with A1241). The pectinesterase/polygalacturonase enzyme blend is sold by the applicant under the proprietary name ROHAPECT® MA Plus. It will be used as a processing aid at low levels and is either not present in the final food or present in insignificant quantities, having no technical function in the final food.

## 1.1 Objectives of the assessment

The objectives of this technical and safety assessment were to:

- determine whether the proposed purpose is a solely technological purpose (function) and that the enzyme achieves its technological purpose as a processing aid in the quantity and form proposed to be used, and
- evaluate potential public health and safety issues that may arise from the use of this enzyme, produced by a genetically modified organism, as a processing aid, specifically by considering the:
  - history of use of the host and gene donor organisms
  - characterisation of the genetic modification(s)
  - safety of the enzyme.

# 2 Food technology assessment

## 2.1 Characterisation of the enzyme

### 2.1.1 Identity of the enzyme

The production microorganism of the enzyme is a GM strain of *A. oryzae*. The donor microorganism of the pectinesterase gene is *A. tubingensis* (further details contained in section 3). The applicant provided relevant information regarding the identity of the pectinesterase enzyme. FSANZ verified this using the IUBMB<sup>2</sup> enzyme nomenclature database (McDonald et al 2009). Details of the identity of the enzyme are provided in Table 1.

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<sup>2</sup> International Union of Biochemistry and Molecular Biology

**Table 1** Identity

<b>Generic common name:</b>	Pectinesterase
<b>Accepted IUBMB name:</b>	Pectinesterase
<b>Systematic name:</b>	Pectin pectylhydrolase
<b>Other names:</b>	Pectin esterase, Pectin demethoxylase; Pectin methoxylase; Pectin methylesterase; Pectase; Pectin methyl esterase; Pectinoesterase
<b>EC number:</b>	3.1.1.11
<b>Reaction:</b>	pectin + <i>n</i> H <sub>2</sub> O = <i>n</i> methanol + pectate

For a graphical representation of the hydrolysis reaction catalysed by pectinesterase, refer to its record in the enzyme database BRENDA<sup>3</sup> (Chang et al 2021).

## 2.2 Manufacturing process

### 2.2.1 Production of the enzyme

AB Enzyme's pectinesterase is produced by submerged fermentation of the genetically modified strain of *A. oryzae*. The main fermentation steps are, inoculum, seed fermentation, main fermentation followed by the recovery stage involving primary and liquid separation, concentration to achieve the desired enzyme activity, polish and germ filtration to provide a concentrated enzyme solution free of the production strain and insoluble substances. This is followed by formulation of the enzyme into an enzyme preparation.<sup>4</sup> AB Enzymes pectinesterase enzyme preparation is sold mainly as a liquid product consisting of glycerol, sodium chloride and water. A manufacturing flow-chart was provided as an Appendix with the application. The production is manufactured in accordance with current Good Manufacturing Practice for Food<sup>5</sup> and the principles of Hazard Analysis and Critical Control Point (HACCP).

The application states that all raw materials used in the fermentation and recovery processes are standard ingredients of food grade quality that meet predefined quality standards. The raw materials conform to either specifications set out in the Food Chemical Codex, 12th edition, 2020 or regulations applying in the European Union. The applicant has advised that a wheat-based material is used during fermentation and is wholly consumed during fermentation. FSANZ considers that it is therefore likely that the wheat-based material in the fermentation media is unlikely to be present in the final commercial enzyme preparation. The Product Data Sheet states absence of cereals containing gluten (i.e. wheat, rye, barley, oats spelt, kamut).

Details on the manufacturing process, raw materials and ingredients used in the production of the pectinesterase enzyme preparation were provided in the application or as Confidential Commercial Information.

<sup>3</sup> [www.brenda-enzymes.org/enzyme.php?ecno=3.1.1.11](http://www.brenda-enzymes.org/enzyme.php?ecno=3.1.1.11)

<sup>4</sup> Enzymes are generally sold as enzyme preparations, which consist of the enzyme(s) and other ingredients, to facilitate their storage, sale, standardisation, dilution or dissolution.

<sup>5</sup> known as cGMP, as distinct from GMP (which refers to the level of use of the enzyme)

## 2.2.2 Specifications

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (FAO/WHO 2006) and in the Food Chemicals Codex (FCC 2008). These specifications are included in earlier publications of the primary sources listed in section S3—2 of Schedule 3 of the Code and enzymes used as a processing aid must meet either of these specifications. The applicant states that the final enzyme preparation complies with the requirements in both of these specifications. Schedule 3 of the Code also includes specifications for arsenic and heavy metals (section S3—4) if they are not already detailed within specifications in sections S3—2 or S3—3.

The applicant provided the results of analysis of three different batches of the pectinesterase preparation. Table 2 provides a comparison of the analyses with international specifications established by JECFA and Food Chemicals Codex, as well as those in the Code (as applicable). Based on these results, and commercial-in-confidence information provided by the applicant, the enzyme preparation met all relevant specifications for arsenic and metals and the microbiological criteria.

**Table 2** Comparison of manufacturer's pectinesterase preparation compared to JECFA, Food Chemicals Codex, and Code specifications for enzymes

Analysis	Analysis provided by manufacturer*	Specifications		
		JECFA (2006)	Food Chemicals Codex (FCC, 2020)	Australia New Zealand Food Standards Code (section S3—4)
Lead (mg/kg)	<0.05	≤ 5	≤ 5	≤2
Arsenic (mg/kg)	<0.5	-	-	≤1
Cadmium (mg/kg)	<0.05	-	-	≤1
Mercury (mg/kg)	<0.05	-	-	≤1
Coliforms (cfu/g)	<30	≤30	≤30	-
Salmonella (in 25 g)	Not detected	Absent	Negative	-
E. coli (in 25 g)	Not detected	Absent	-	-
Antibiotic activity	Not detected	Absent	-	-

\* across three samples

Whilst the manufacturing processes ensure the production microorganism is removed from the final enzyme preparation, the food enzyme is a biological isolate of variable composition, containing the enzyme protein, as well as organic and inorganic material derived from the microorganism and fermentation process. Refer to section 3.4 below for the total organic solids (TOS) value.

## 2.3 Technological purpose of the enzyme

Under the current application, pectinesterase is intended for use to in the production of a number of specific products (refer to section 3.2.1 below).

Pectinesterase is a hydrolase enzyme that catalyses the de-esterification of methyl ester linkages of the galacturonan backbone of pectic substances to release acidic pectins (pectate/pectic acid) and methanol. It is, therefore, a pectin-degrading enzyme (along with pectin lyases and polygalacturonase). These enzymes are often collectively called 'pectinases' and are typically found in plants and microorganisms (especially fungi). They are a mixture of enzymes that act on pectic substances (plant polysaccharides that maintain the integrity of the cell wall or middle lamella).

The result of pectinesterase action is de-esterification of the 'smooth' region pectin converting the pectin into a partially demethylated version or pectic acid. As stated in the application, the practical outcome of such activity is that intercellular barriers (middle lamella) are broken down.

The application describes the two classes of pectinases – namely pectin depolymerases (further classified as polygalacturonases and pectin lyases) and pectinesterases. Polygalacturonase (under A1240) is a pectin depolymerase (it splits the main chain), whereas pectinesterase has the ability to de-esterify pectin by the removal of methoxy residues. Pectinesterase causes a reduction in viscosity of a pectic substrate. As explained in the application, several enzymes are sometimes used simultaneously, to achieve complete pectin degradation.

The stated technological purpose of the pectinesterase enzyme is supported by scientific literature (e.g. Damodaran et al, 2008; Nagodawithana and Reed, 1993).

The applicant provided information on the physical and chemical properties of the enzyme preparation. Table 3 summarises this information.

**Table 3** *Physical and chemical properties of pectinesterase enzyme preparation*

Physical and chemical properties of commercial enzyme preparation	
Enzyme activity	Minimum pectinesterase activity 10,900 PE/g* (from three batches)
Appearance	Brown coloured liquid
Storage conditions	Store below 10°C.
Storage stability	Three year shelf life at 10°C
Density	1.1 g/ml

\*Assay of pectinesterase activity (AB Enzymes internal method)

Pectinesterase (from two sources) is approved for use in the manufacture of foods since it is listed in subsection S18—4(5).

## 2.4 Technological justification

As outlined above, the technological need of the enzymatic conversion of pectin with the help of pectinesterase can be described as enabling the degradation of pectin. Pectin causes technical difficulties during food production due to its high viscosity and gelling properties in processing raw materials that contain this component.

The enzyme performs its function of catalysing the hydrolysis of pectic substances during the production of plant foods. It is therefore performing as a processing aid as defined by the Code.

The Code already permits pectinesterase and polygalacturonase (from other sources) to be used in the manufacture of foods, the two enzymes the applicant combines in its commercial product ROHAPECT® MA Plus. The specific benefits of the action of pectinesterase in the manufacture and/or processing of fruit and vegetable juices/products; and in the production of coffee, flavouring substances and wine, as summarised from the application, are described below.

The Codex guideline, *Guidelines on Substances used as Processing Aids* (CAC/GL 75-2010) sets out general principles for the safe use of substances used as processing aids. The Guideline states that substances used as processing aids shall be used under conditions of good manufacturing practice (GMP). Therefore use of commercial enzyme preparations should follow GMP, where use is at a level that is not higher than that necessary to achieve the desired enzymatic reaction. The applicant requested use of the enzyme at GMP levels.

#### Fruit and vegetable manufacture and/or processing (fruit juices/products and vegetable juices/products)

Pectinesterase assists in the degradation of pectin in processing of these products. While fruits and vegetables naturally contain pectinesterase (hence the ripening of fruit and vegetables), the concentration is too variable and the specificity of the naturally occurring enzyme may not be optimal for the desired production process.

#### Coffee production

Pectinases, including pectinesterase, are used during coffee processing for processing improvement such as to assist with separation of the bean from the outer layers and a shorter fermentation time.

#### Flavouring substances production

Flavouring substances used to flavour foods can be produced using enzymes (such as cellulases and pectinases). Enzymatic pre-treatment for the extraction of flavour components from various plant materials enhance aroma recovery.

#### Wine production

Grapes have a high pectin content, and pectinases are already used in wine making. They are used in a number of stages in the wine making process, as described in the application. For example, when pectinesterase is used in combination with polygalacturonase in red wine making, the visual aspects (e.g. colour, stability) are improved compared to untreated wines.

## **2.5 Food technology conclusion**

FSANZ concludes that the proposed use of this pectinesterase in the production of a number of foods is consistent with its typical function as a pectinase. The evidence presented provides adequate assurance that the use of the enzyme, in the form and requested amount (i.e. at a level consistent with GMP) is technologically justified and effective in achieving its stated purpose.

Pectinesterase performs its technological purpose during the production of food and is not performing a technological purpose in the final food. It is therefore appropriately categorised as a processing aid as defined in the Code.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that the enzyme meets these specifications.



## 3 Safety assessment

Some information relevant to this section is Confidential Commercial Information (CCI), so full details have not been provided in this public report.

### 3.1 Source microorganisms

#### 3.1.1 Host organism

The enzyme production strain, *A. oryzae* AR-962, is derived from a parental strain of *A. oryzae* collected in South America in 1984.

While *A. oryzae* has been implicated in illness in severely ill and/or immunocompromised individuals, it is not considered to be pathogenic in healthy humans (Barbesgaard et al 1992). It is listed as a Risk Group 1 microorganism by the German Federal Office of Consumer Protection and Food Safety.<sup>6</sup> Evidence was also provided that the enzyme production strain was not detected in the liquid enzyme concentrate.

*A. oryzae* is generally considered to be a domesticated form of *A. flavus*—a species which includes many aflatoxin-producing strains—and it can be difficult to differentiate between the two (Frisvad et al 2018). The applicant provided commercial-in-confidence evidence that adequately demonstrated that the production strain was correctly identified as *A. oryzae*. Data was provided to show that it did not produce toxicologically significant amounts of mycotoxins. Three different batches of liquid enzyme concentrate—ie concentrated, filtered fermentation culture supernatant—showed levels of aflatoxins below 0.1 ug/kg.

Taking into consideration evidence provided, and the history of safe use of *A. oryzae* for food and enzyme production, it is concluded that the enzyme production strain *A. oryzae* AR-962 is non-pathogenic, non-toxigenic, and does not present an unacceptable food safety risk.

#### 3.1.2 Gene donor organisms

The gene for the pectinesterase enzyme was isolated from *A. tubingensis*, a filamentous fungus belonging to *Aspergillus* section Nigri (the black aspergilli; Samson et al. 2006). *A. tubingensis* has been implicated in skin, ear and respiratory infections in humans (Gautier et al. 2016). However, as the pectinesterase gene has been manipulated through standard DNA cloning methods, and extraneous material from *A. tubingensis* would not be carried across to the enzyme production organism, no food safety hazard is identified.

### 3.2 Characterisation of the genetic modification(s)

#### 3.2.1 Description of the DNA to be introduced and method of transformation

An expression cassette containing the pectinesterase gene was introduced into a spontaneous mutant host strain of *A. oryzae*, producing the AR-962 production strain. The pectinesterase gene is derived from *A. tubingensis* and is under the control of a promoter and terminator. Data provided by the applicant and analysed by FSANZ confirmed the identity of the pectinesterase enzyme.

A selection cassette containing the acetamidase gene (*amdS*) from *Aspergillus nidulans*

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<sup>6</sup>

[www.bvl.bund.de/SharedDocs/Downloads/06\\_Gentechnik/register\\_datenbanken/organismenliste\\_xls.html;jsessionid=F8D7A39D1FED4DD1FA972AA6A8FEC7F6.1\\_cid363](http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/register_datenbanken/organismenliste_xls.html;jsessionid=F8D7A39D1FED4DD1FA972AA6A8FEC7F6.1_cid363)

(*A. nidulans*) was also introduced into the host strain (Kelly and Hynes, 1985). The *amdS* gene allows for selection of transformants on acetamide-containing media. Acetamide has been widely used as a selection marker in fungal transformations. The inclusion of this selection marker is standard in filamentous fungi systems producing a variety of recombinant gene products (Gryshyna et al., 2016) and there are no safety concerns.

The introduced DNA was integrated into the genome of the host strain through standard transformation techniques using protoplasts. While plasmids were used in the construction of the expression and selection cassettes, no plasmid derived sequences were integrated.

### **3.2.2 Characterisation of inserted DNA**

Southern blot analysis was used to characterise the inserted DNA in the AR-962 production strain. The results indicate the presence of the inserted DNA in the genome of AR-962.

Due to the transformation method, no antibiotic resistance genes were introduced into the AR-962 production strain.

No recombinant DNA was detected in the liquid enzyme concentrate.

### **3.2.3 Genetic stability of the inserted gene**

The assessment confirmed the inserted DNA is integrated into the production organism's genome and does not have the ability to replicate autonomously. The inserted gene is therefore considered to be genetically stable.

To provide further evidence of the stability of the introduced pectinesterase gene the applicant provided phenotypic data demonstrating that the enzyme is expressed over successive generations.

The applicant examined the activity level of the pectinesterase enzyme in a number of fermentation batches. These data confirmed that the pectinesterase gene is expressed over multiple generations and is stable.

## **3.3 Safety of the pectinesterase enzyme**

### **3.3.1 History of safe use of pectinesterase**

Pectinesterase synthesized by the donor organism, *A. tubingensis*, is approved in the Code (Schedule 18) and has also been approved by other regulatory agencies. The specific enzyme that is the subject of this application has been approved for use in Denmark and France, and confidential information was provided by the applicant to show that substantial quantities are sold commercially.

### **3.3.2 Bioinformatics concerning potential for toxicity**

A recent (August 2020) homology search, using the amino acid sequence of the pectinesterase as the query sequence, was performed on proteins marked as toxins in the NCBI Identical Protein Groups (IPG) database using BLAST-P, which is a basic local alignment search tool. Search results were provided to FSANZ to show that the pectinesterase enzyme does not show significant homology to the sequence of any protein known to be a toxin.

### 3.3.3 Toxicology data

Toxicology studies conducted with the pectinesterase that is the subject of this application include a 13-week repeat-dose oral gavage study in rats, and two genotoxicity studies; a bacterial reverse mutation assay (Ames test) and an *in vitro* micronucleus assay.

#### 3.3.3.1 Short term study in animals

*Thirteen-week repeat-dose oral gavage study of pectinesterase in Wistar rats (Schmid et al 2020; unpublished study). Regulatory status: GLP; in compliance with OECD test guideline 408*

The control article and vehicle for this study was sterile water for injection. Stability and homogeneity of the test article in this vehicle were verified. Rats, 10/sex/group, were group housed under standard laboratory conditions of environment and husbandry. They were gavaged daily, for 90 days, with 0, 100, 300 or 1000 mg TOS<sup>7</sup>/kg bw/day. Parameters measured included survival, clinical observations, bodyweight changes, food consumption, performance on a functional observational battery, clinical pathology parameters (endocrine, haematology, coagulation, clinical chemistry, urinalysis), gross necropsy findings, weights of selected organs, and histopathology of a comprehensive list of organs and tissues.

All rats survived to the end of the in-life phase. There were no treatment-related effects on any parameters, with the exception of clinical observations consistent with low palatability of the  $\geq 300$  mg/kg bw dose formulations, including salivation and moving bedding. These clinical signs were not regarded as being evidence of toxicity.

It was concluded that the No Observed Adverse Effect Level (NOAEL) of pectinesterase synthesized by GM *A. oryzae* is 1000 mg TOS/kg bw/day, the highest dose tested.

#### 3.3.3.2 Genotoxicity studies

*Bacterial reverse mutation assay (Schreib et al 2019; unpublished study). Regulatory status: GLP; in compliance with OECD Guideline 471*

The test system for this assay comprised the *Salmonella enterica* ser. Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102. The solvent and negative control article was distilled water. Appropriate positive control articles, as recommended in the Guideline, were used. Two separate experiments were conducted. The plate incorporation method was used in Experiment I, and the pre-incubation method was used in Experiment II. Each test was conducted in triplicate, with and without addition of S9 mix for metabolic activation. In both Experiment I and Experiment II, concentrations of pectinesterase tested were 31.6, 100, 316, 1000, 2500 and 5000  $\mu\text{g}/\text{plate}$ . No precipitation of test article was observed in either experiment. Evidence of cytotoxicity was limited to concentrations of pectinesterase  $\geq 2500$   $\mu\text{g}/\text{plate}$ , in the absence of S9 mix.

No biologically relevant increases in revertant colony numbers of any of the *S. typhimurium* strains were observed following treatment with pectinesterase at any concentration, with or without S9 mix, in either experiment. The positive control articles induced the expected significant increases in numbers of revertant colonies in both experiments, confirming the validity of the assay. It was concluded that pectinesterase is not mutagenic under the conditions of the assay.

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<sup>7</sup> TOS = Total Organic Solids

*In vitro micronuclei assay (Donath et al 2019; unpublished study). Regulatory status: GLP; in compliance with OECD Guideline 473*

The test system for this study comprised human lymphocytes harvested from the peripheral blood of healthy non-smoking donors of unspecified sex.

Test article concentrations for the definitive experiments, based on results of the dose-range-finding experiment, were 500, 600 and 700 µg/mL without S9 mix and 500, 700 and 900 µg/mL with S9 mix in the 4-hour exposure protocol (Experiment I). In the 44-hour exposure protocol (Experiment II), the test article concentrations were 50, 100 and 150 µg/mL. Appropriate positive control articles, as recommended by the Guideline, and negative control (culture medium) were also assayed.

No precipitation of the test article was observed at any concentration in either experiment, but some evidence of cytotoxicity was observed. In Experiment I, increased cytostasis was observed at ≥ 600 µg/mL without S9 and at ≥700 µg/mL with S9. In Experiment II an increase in cytostasis was observed at 150 µg/mL.

No statistically significant increase in the frequency of micronucleated cells was noted after treatment with the test article in either experiment I and II. The positive control articles induced the expected statistically significant increases in the frequencies of micronuclei, confirming the validity of the assay.

It was concluded that pectinesterase did not induce structural and/or numerical chromosomal damage in human lymphocytes, and is considered to be non-mutagenic in the *in vitro* mammalian cell micronucleus test.

### **3.3.4 Potential for allergenicity**

The results of recent (2020) searches of the FARRP<sup>8</sup> and SDAP<sup>9</sup> databases for homology with the amino acid sequence of the pectinesterase were provided. Searches were for alignment of the entire amino acid sequence and alignment of sliding 80-amino acid windows of the protein to known protein allergens. The identity percentages of all the hits from both FARRP and SDAP were below the set 35 % identity limit. Using the 80 amino acid sliding window search, the enzyme sequence was found to have degrees of identity from 36.3% to 38.8 % with pollen allergens of two plant species, a common weed *Salsola kali*, and the olive tree, *Olea europaea*. These pollen allergens are respiratory allergens rather than food allergens, and there is good evidence that respiratory allergens do not represent an allergic hazard when consumed (Bindslev-Jensen et al 2006). The predictive value of the 80 amino acid sliding window search has been called in to question by Ladics et al. (2007) and Goodman and Tetteh (2011), with the latter authors recommending that degree of identity less than 50% should not be considered relevant. As noted in Section 2.2.1 above, wheat-based products are used in the fermentation media, however they are likely to be consumed during the fermentation process. Given the absence of homology of the enzyme with known food allergens, and that allergens from wheat are unlikely to be present in the commercial preparation, FSANZ concludes that the commercial enzyme preparation is unlikely to pose a risk of food allergy.

### **3.3.5 Assessments by other regulatory agencies**

No safety assessments by other regulatory agencies are available.

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<sup>8</sup> <http://allergenonline.org>

<sup>9</sup> <https://fermi.utmb.edu/>

### 3.4 Dietary exposure assessment

The objective of the dietary exposure assessment was to review the budget method calculation presented by the applicant as a 'worse-case scenario' approach to estimating likely levels of dietary exposure assuming all added pectinesterase enzyme from GM *A. oryzae* remained in the food.

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass *et al.*, 1997). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive. The TMDI can then be compared to an acceptable daily intake (ADI) or a NOAEL to estimate a margin of exposure for risk characterisation purposes.

In their budget method calculation, the applicant made the following assumptions:

- the maximum physiological requirement of solid foods (including milk) is 25 g/kg body weight/day
- the maximum physiological requirement for non-milk beverages is 100 mL/kg body weight/day (the standard level used in a budget method calculation)
- 50% of solid foods and 25% of non-milk beverages contain pectinesterase
- the maximum pectinesterase level in final solid foods was 26.0 mg TOS/kg food and for non-milk beverages was 2.6 mg TOS/kg food (i.e. the highest use level from all uses within each group)
- all of the enzyme remains in the final food.

Based on these assumptions, the applicant calculated the TMDI of pectinesterase to be 0.390 mg TOS/kg body weight/day.

As assumptions made by the applicant differ to those that FSANZ would have made in applying the budget method, FSANZ independently calculated the TMDI using the following different assumptions that are conservative and reflective of a first tier in estimating dietary exposure:

- the maximum physiological requirement for solid foods (including milk) is 50 g/kg body weight/day. This is the standard level used in a budget method calculation where there is potential for the enzyme to be in baby foods or general purpose foods that would be consumed by infants (Hansen, 1966), which for this enzyme would be from processed fruits and vegetables (e.g. canned fruits, canned vegetables and jams), soups, sauces, bouillons, dressings, condiments, snack foods, meat-derived foods and breads/crackers.
- FSANZ would generally assume 12.5% of solid foods contain the enzyme based on commonly used default proportions noted in the FAO/WHO Environmental Health Criteria (EHC) 240 Chapter 6 on dietary exposure assessment (FAO/WHO 2009). However the applicant has assumed a higher proportion of 50% based on the nature and extent of use of the enzyme and therefore FSANZ has also used this proportion as a worst case scenario.

All other inputs and assumptions used by FSANZ remained as per those used by the applicant. The TMDI based on FSANZ's calculations for solid foods and non-milk beverages were 0.650 mg TOS/kg body weight/day and 0.065 mg TOS/kg body weight/day respectively, resulting in a total of 0.715 mg TOS/kg bw/day.

Both the FSANZ and applicant's estimates of the TMDI will be overestimates of the dietary exposure given the conservatism in the budget method. This includes that it was assumed that the enzyme remains in the final foods and beverages. The applicant has stated that the enzyme is inactivated by heat or removed during further processing steps and does not have a function in the final food.

## 4 Discussion

AB Enzyme's pectinesterase is produced by a genetically modified strain of *A. oryzae*. It is intended for use as a processing aid in the manufacture and/or processing of fruit and vegetable juices/products; and in the production of coffee, flavouring substances and wine.

The production strain contains an expression cassette—containing the pectinesterase gene from *A. tubingensis* and the acetamidase gene from *A. nidulans* as a selectable marker—integrated into its genome.

The enzyme is produced by submerged fermentation of the production strain, followed by recovery and clean-up from the fermentation medium in accordance with principles of cGMP and HACCP.

Evidence provided confirms that the production strain is neither pathogenic nor toxigenic, and analysis of the activity level of the pectinesterase enzyme in a number of fermentation batches over multiple generations confirmed the presence and stability of the introduced DNA.

Toxicity testing of the enzyme showed no evidence of genotoxicity *in vitro*. The NOAEL in a 13-week oral gavage study in rats was 1000 mg TOS/kg bw/day, which was the highest dose tested. The TMDI was calculated by FSANZ to be 0.715 mg TOS/kg bw/day. Comparison of the NOAEL and the TMDI gives a margin of exposure (MOE) of approximately 1400.

Bioinformatic analysis indicated that the enzyme shows no significant homology with any known toxins or venoms. A degree of homology between the enzyme and two pollen allergens was found. Taking into account that neither of these allergens is a food allergen and that only low levels of the enzyme processing aid are expected to be present in final food products, the risk of food allergy from the proposed uses of the enzyme is likely to be low.

## 5 Conclusion

FSANZ concludes that the proposed use of this pectinesterase is technologically justified and has been demonstrated to be effective in achieving its stated purpose. The applicant requested use at GMP levels.

Pectinesterase performs its technological purpose during the production of food and is not performing a technological purpose in the final food. It is, therefore, appropriately categorised as a processing aid as defined in the Code.

There are relevant identity and purity specifications for the enzyme in the Code and the applicant provided evidence that the enzyme meets these specifications.

The enzyme production strain *A. oryzae* AR-962 is non-pathogenic and non-toxigenic, and does not present an unacceptable food safety risk. No food safety hazard was identified in the isolation and use of the pectinesterase gene from *A. tubingensis*, and there are no safety concerns with the use of the acetamidase selectable marker gene from *A. nidulans*.

No public health and safety concerns were identified in the assessment of enzyme under the proposed use conditions. Based on the reviewed data it is concluded that, in the absence of any identifiable hazard, an ADI 'not specified' is appropriate.

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