

21 September 2016
[23–16]

Supporting document 1

Risk and technical assessment report – Application A1121

Oryzin (Protease) from *Aspergillus melleus* as a Processing Aid (Enzyme)

Executive summary

Application A1121 seeks approval to use the enzyme oryzin, sourced from a mutated strain of *Aspergillus melleus* (strain P-52), as a processing aid. Oryzin is a serine endopeptidase which can catalyse the hydrolysis of proteins with broad specificity, but does not hydrolyse peptide amides. The Applicant states that the enzyme will be used in flavourings, cereal products produced from flour, enzyme modified cheese or dairy ingredients used in other foods, processed egg products, and in products such as dressings and sauces, meat and fish extracts, and protein hydrolysates and yeast extracts used in other foods.

The evidence presented to support the proposed uses provides adequate assurance that the enzyme, in the form and prescribed amounts, is technologically justified to be effective in achieving its stated purpose. The enzyme preparation meets international purity specifications for enzymes used in the production of food.

There are no public health and safety issues associated with the use of the enzyme preparation containing oryzin produced by mutated *A. melleus* strain P-52, as a food processing aid on the basis of the following considerations:

- The wild type strain has been characterised as *A. melleus* and is deposited at the Japan Collection of Microorganisms.
- The production organism is not toxigenic or pathogenic. Further, *A. melleus* has a long history of safe use overseas as the production organism for a number of processing aids.
- Residual oryzin is expected to be present in the final food but recommended conditions for use would render the enzyme inactive and it would be susceptible to digestion like any other dietary protein.
- Bioinformatic analysis indicated that oryzin has no biologically relevant homology to known food protein allergens.

- The oryzin preparation caused no observable effects at the highest tested doses in a 26-week repeated dose toxicity study in rats. The No Observable Adverse Effect Level (NOAEL) for the oryzin concentrate was determined to be 2000 mg/kg body weight/day for male rats.
- The enzyme was not genotoxic *in vitro*.

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

Table of contents

EXECUTIVE SUMMARY	1
1 INTRODUCTION	2
1.1 OBJECTIVES OF THE RISK AND TECHNICAL ASSESSMENT	2
2 FOOD TECHNOLOGY ASSESSMENT	2
2.1 CHARACTERISATION OF ORYZIN	2
2.1.1 <i>Identity of the enzyme</i>	2
2.1.2 <i>Enzymatic properties</i>	3
2.1.3 <i>Physical properties</i>	4
2.2 PRODUCTION OF THE ENZYME.....	4
2.2.1 <i>Potential presence of allergens</i>	4
2.3 SPECIFICATIONS	4
2.4 TECHNOLOGICAL FUNCTION.....	5
<i>Baking</i>	5
<i>Dairy processing</i>	5
<i>Egg processing</i>	5
<i>Meat and fish processing</i>	5
<i>Protein and yeast processing</i>	5
<i>Flavour production</i>	6
2.5 FOOD TECHNOLOGY CONCLUSION.....	6
3 HAZARD ASSESSMENT	6
3.1 SCOPE OF THE ASSESSMENT	6
3.2 HAZARD OF THE PRODUCTION ORGANISM – <i>A. MELLEUS</i> STRAIN P-52.....	6
3.3 HAZARD OF THE ENZYME ORYZIN	7
3.3.1 <i>History of Use</i>	7
3.3.2 <i>Bioinformatic analysis for potential allergenicity</i>	7
3.4 EVALUATION OF TOXICITY STUDIES OF THE ENZYME PRODUCT	8
3.4.1 <i>Sub-chronic toxicity</i>	8
3.4.2 <i>Genotoxicity</i>	9
3.4.3 <i>Residual allergens and substances causing food intolerance from the culture medium</i> ..	11
3.5 RISK ASSESSMENT CONCLUSIONS.....	11
REFERENCES	12

1 Introduction

FSANZ received an application from Amano Enzyme Inc. Japan seeking approval for the enzyme oryzin (EC 3.4.21.63) as a processing aid. The Applicant states the enzyme will be used in flavourings, cereal products produced from flour, enzyme modified cheese or dairy ingredients used in other foods, processed egg products, and in products such as dressings and sauces, meat and fish extracts, and protein hydrolysates and yeast extracts used in other foods. The enzyme is sourced from a chemically mutated strain of *Aspergillus melleus*, strain P-52. Oryzin is produced from this fungal species by means of a fermentation process.

Oryzin is characterised as a serine endopeptidase (or serine protease). Proteases catalyse the hydrolysis of peptide bonds in proteins and polyphenols. Oryzin can catalyse the hydrolysis of proteins with broad specificity, but does not hydrolyse peptide amides. During food processing, the use of oryzin results in improvement of organoleptic properties (taste and flavour), physiological properties (foamability, emulsifying ability, heat stability and viscosity) and nutritional properties (absorptivity and digestibility).

1.1 Objectives of the risk and technical assessment

Currently, there are no permissions for the enzyme oryzin from *A. melleus* or any other source in the Code. Therefore, any application to amend the Code to permit the use of this enzyme as a food processing aid requires a pre-market assessment.

The objectives of this risk assessment were to:

- determine whether the proposed purpose is clearly stated and that the enzyme achieves its technological function in the quantity and form proposed to be used as a food processing aid
- evaluate any potential public health and safety concerns that may arise from the use of oryzin as a processing aid.

2 Food Technology Assessment

2.1 Characterisation of oryzin

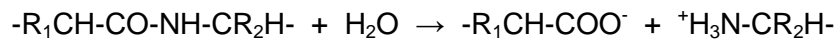
2.1.1 Identity of the enzyme

Information regarding the identity of the enzyme provided by the Application has been verified using the appropriate internationally accepted reference for enzyme nomenclature, the International Union of Biology and Molecular Biology (IUBMB 2016). Additional information located from the IUBMB has also been included.

Generic common name	Protease
Accepted IUBMB name	Oryzin
EC ¹ number	3.4.21.63
CAS number	9074-07-1
Commercial name	Protease P “Amano” 6SD/K or Protease P “Amano” 6SD

¹ EC: Enzyme Commission, internationally recognised number that provides a unique identifier for the enzyme

Reaction: Hydrolysis of proteins with broad specificity



2.1.2 Enzymatic properties

Oryzin hydrolyses proteins and has broad specificity. However, the enzyme does not hydrolyse peptide amides.

The peak activity of oryzin occurs in the temperature range of 40-45°C, being stable up to 50°C and inactivated at temperatures above 55°C (see Figure 1).

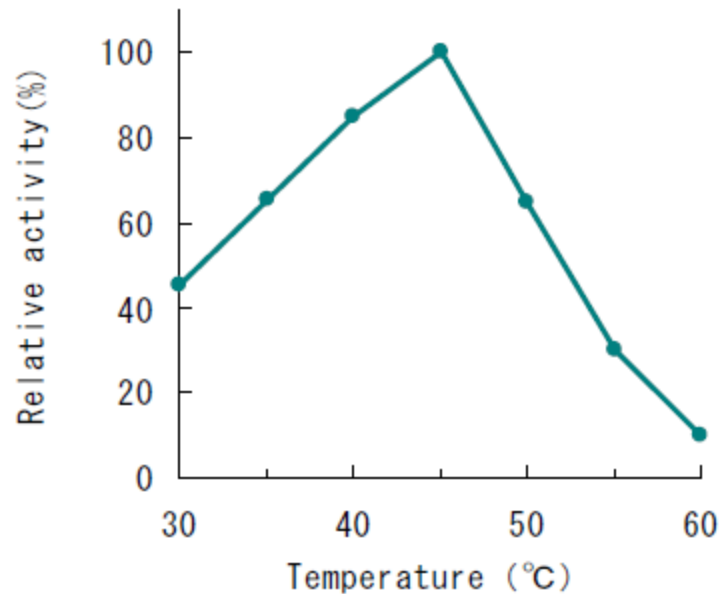


Figure 1: Effect of temperature on the enzyme activity (taken from the Application)

The peak activity of oryzin occurs in the pH range of 7-8, being stable in the pH range of 4-11, and being inactivated below pH 4 or above pH 11 (see Figure 2).

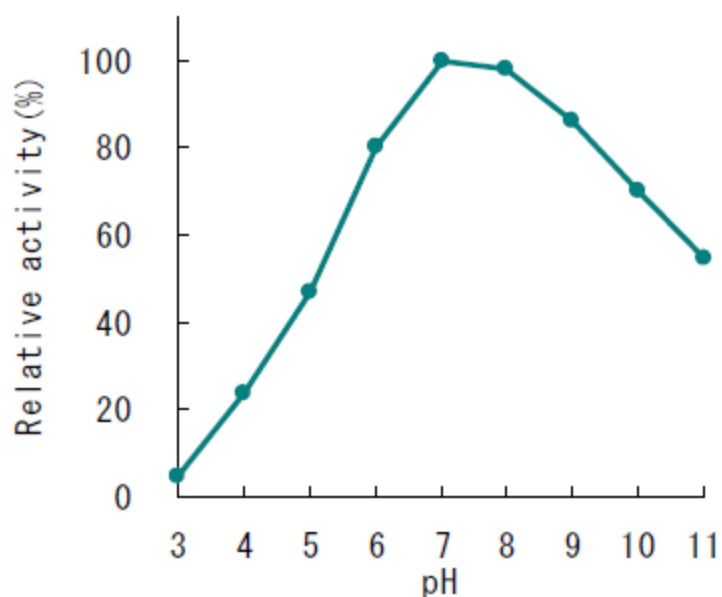


Figure 2: Effect of pH on the enzyme activity (taken from the Application)

The Application states that the oryzin enzyme preparation does not possess any significant secondary activities.

Stored samples of the oryzin enzyme preparation kept in an airtight bag at room temperature were tested for activity by the Applicant and were shown to have maintained good activity for 24 months confirming it is quite stable for this period of time.

2.1.3 Physical properties

The oryzin enzyme preparation is a dried powder with potato dextrin used as the enzyme carrier and diluent.

2.2 Production of the enzyme

The enzyme is produced using the standard fermentation process which is common for the production of many food enzymes.

The production steps are summarised as fermentation under standard culturing conditions, extraction of the enzyme from the fermentation media, separation and concentration, filtration and then formulation into the commercial enzyme preparation.

The fermentation process involves two steps; the initial inoculum fermentations to produce enough of the microorganism, and then the main production fermentation. The next steps post fermentation are removal of the enzyme from the fermentation media, filtration to remove the production strain and other solids, ultrafiltration to further purify and concentrate the enzyme preparation, spray drying to produce the enzyme preparation as a powder using potato dextrin as the solid support and carrier. Finally the preparation is standardised to the commercial enzyme activity and packaged for sale.

All the raw materials used in the production of the enzyme preparation are permitted food additives and processing aids in the Code (as detailed in the Application) and are appropriate for their purpose.

2.2.1 Potential presence of allergens

The Application notes that soy and wheat products (flour and bran) are used in the fermentation media. However, the Application also states that 'residual soy and wheat allergens are not present in Oryzin (Protease) enzyme powder (less than 3.0µg/g)'.

Dextrin is used as the enzyme carrier in the enzyme preparation but it is sourced from potato, which does not require mandatory declaration on a label.

2.3 Specifications

There are international specifications for enzyme preparations used in food production. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2006) and the Food Chemical Codex (U.S. Pharmacopeial Convention 2015). Both of these specification sources are primary sources listed in Schedule 3 (Identity and Purity), specifically section S3—2. Enzyme preparations need to meet these enzyme specifications.

The Application provides analytical results on oryzin batches confirming the enzyme preparation meets these international specifications (see Table 2.1).

Table 2.1: Enzyme preparation compared to the JECFA and Food Chemicals Codex (FCC) enzyme specifications

Analysis	Specifications		
	Product	JECFA	FCC
Lead (mg/kg)	≤2	≤5	≤5
Coliforms (cfu/g)	≤10	≤30	≤30
<i>E. coli</i>	ND/10 g	ND/25 g	
<i>Salmonella</i> (in 25 g)	ND	ND	ND
Antibiotic activity	ND	ND	

ND Not detected

2.4 Technological function

Protease enzymes in general are used in a wide variety of food processing applications. There are several hundred proteases that have commercial relevance. Oryzin comes under the general family of protease enzymes, and specifically the enzyme family called serine endopeptidases with the EC number 3.4.21.XX. Proteases catalyse the hydrolysis of peptide bonds in proteins and polyphenols, but oryzin does not hydrolyse peptide amides.

Information provided by the Applicant supports the claim that oryzin (as a protease enzyme) can be used for food processing in a variety of food categories. The examples provided are baking, dairy, egg, meat and fish, and protein processing, as well as flavour production. The final foods produced using the enzyme are stated to be flavourings, cereal products produced from flour, enzyme modified cheese or dairy ingredients used in other foods, processed egg products used in products such as dressings and sauces, meat and fish extracts, and protein hydrolysates and yeast extracts used in other foods.

Baking

The Applicant claims use of the enzyme can improve the strength of the gluten network, to provide a larger loaf volume and improved texture.

Dairy processing

The Applicant claims the enzyme can be used to produce both enzyme modified cheese and enzyme modified butter, both dairy products that can be used to add specific dairy type cheese and butter flavours to foods. The enzyme is used to accelerate the ripening of these products starting with cheese and butter or butter style starting ingredients. Use of the enzyme can change the final amino acid composition, and hence flavour and odour of the final ingredients.

Egg processing

The Applicant claims that using the enzyme in the processing of egg products can strengthen the egg flavour, improve the heat tolerance and limit the tendency to congeal when heated.

Meat and fish processing

Using the enzyme during processing to produce meat and fish extracts is claimed to improve the taste, yield, nutritional content and physical properties.

Protein and yeast processing

Protein processing refers to the production of highly flavoured protein products such as hydrolysed vegetable protein which are used as amino-acid based seasoning ingredients in food.

Enzymes are commonly used for the production of such products and oryzin is claimed to be such an enzyme. The enzyme can also be used to produce yeast extract products which also provide flavour and taste when added to other foods.

Flavour production

In a similar way to protein and yeast processing to produce highly flavoured extracts, oryzin can be used for enzymatic flavour production.

2.5 Food technology conclusion

The evidence presented to support the proposed uses of the oryzin enzyme preparation in food production to hydrolyse proteins provided adequate assurance that the enzyme is technologically justified. The enzyme preparation meets international purity specifications.

3 Hazard Assessment

3.1 Scope of the assessment

The hazard of oryzin derived from *A. melleus* was evaluated by considering:

- the hazard of the production organism, including any history of safe use in food production processes
- the hazard of the encoded protein, including potential allergenicity
- the toxicity studies on the enzyme preparation intended for commercial use.

3.2 Hazard of the production organism – *A. melleus* strain P-52

The Applicant has provided information on the parental lineage of the production organism, which is classified as *A. melleus* strain no.26st, a wild type isolated from Japanese soil. This isolate is deposited with the Japan Collection of Microorganisms as *A. melleus* IAM2066. The production strain P-52 is a mutant derived from strain no.26st through numerous rounds of mono spore isolation and mutagenesis using *N*-methyl-*N'*-nitrosoguanidine, UV radiation and ⁶⁰Cobalt. Strain P-52 is not genetically modified and has been confirmed as belonging to the species *A. melleus*.

A. melleus has not been previously assessed by FSANZ. However, *A. melleus* has been used for many years for food or feed purposes or in the production of enzymes used as processing aids in Denmark, France, Canada, Japan and China. The Applicant has used the *A. melleus* in this submission for over 35 years for the production of food enzymes, with no reports of pathogenicity or toxicity to the workers exposed to this strain.

The Applicant conducted a literature search on the pathogenicity and toxicity of the genus *Aspergillus* and the species *A. melleus*.

The search showed that some *Aspergillus* species can occasionally cause infections in humans, but this has been rare and most commonly reported in immunosuppressed patients (Kauffman 2006).

However, pathogenicity in humans was not reported for *A. melleus*. Strain P-52 was tested for toxicologically significant amounts of mycotoxins such as Aflatoxins B1, B2, G1 and G2; Ochratoxin A; HT-2 Toxin (HT-2); T-2 Toxin (T2); Sterigmatocystin; Fumonisin B1 and B2; and Zearalenone. None of these mycotoxins were detected.

A. melleus is considered a safe strain for production of enzyme preparations used in food processing (Pariza and Johnson, 2001). Protease derived from *A. melleus* was marketed as a digestive aid in North America prior to 1994 (ETA, 2015). Further, protease derived from *A. melleus* is included on the Association of Manufacturers and Formulators of Enzyme Products (AMFEP, 2015) list of commercialised enzymes.

The Applicant has indicated that in order to ensure the genetic stability of the enzyme, it is produced under well controlled manufacturing processes which are in compliance with AMFEP's guidelines for the safe handling of microbial enzyme preparations.

The enzyme is produced according to the FSSC22000 quality control system and complies with international guidelines for the safe handling of microbial enzyme preparations published by the AMFEP. Certification of Good Manufacturing Practices (GMP) for food additives and conformity to the FSSC22000 quality control system, have been provided by the Applicant.

3.3 Hazard of the enzyme oryzin

3.3.1 History of Use

The Applicant has indicated their safe use of *A. melleus* for the production of food enzymes for over 35 years. Oryzin derived from *A. melleus* has been approved for use overseas (see Call for Submission report Section 1.3.2), and the oryzin enzyme preparation meets international specifications for chemical and microbiological purity of food enzymes (see Section 2.3).

3.3.2 Bioinformatic analysis for potential allergenicity

Protease activity is a common characteristic of many allergens. A large number of mould (fungi) species are known to produce proteases, with alkaline serine proteases the major allergens of *Aspergillus* species (Matsumura, 2012).

The Applicant has provided the results of an *in silico* analysis comparing the oryzin sequence from *A. melleus* with a database of known allergens. The sequence was compared to identify matches for 6-consecutive amino acids and an 80 amino acid sliding window (with 35% or higher identities within the 80 amino acid stretch). There were 19 hits (matches) in the search for 6-consecutive amino acids and 24 hits for the 80 amino acid sliding window with greater than 35% identity with oryzin. The Applicant has indicated that these known allergens cause an allergic response mainly via inhalation (Matsumura, 2012) and sometimes by contact and hence are not identified as food allergens.

3.4 Evaluation of toxicity studies of the enzyme product

3.4.1 Sub-chronic toxicity

Individual unpublished study

Tamura, S., Katoh, S., Sakuma, N., and Ogawa, S. (1974) Toxicity test of protease P "Amano" 6. Tokyo Dental College and Amano Pharmaceutical Co., Ltd., Japan.

Acute toxicity study

Male and female mice (ddy/S strain) and rats (Wistar strain) were assigned to groups of 10 animals per sex per species per treatment. Feed (CE-2, NIPPON CLEA) and tap water were given *ad libitum*. The enzyme preparation (Protease P "Amano" 6 comprising oryzin concentrate diluted with potato dextrin; Lot No PZ4810N, with protease activity of 806,000 µ/g) was administered via three methods: oral (gavage), subcutaneous and intraperitoneal (both by injection).

The amount of protease administered ranged from 10,000–25,000 mg/kg body weight (bw), 37–320 mg/kg bw and 34.4–225.6 mg/kg bw for the oral, subcutaneous and intraperitoneal methods, respectively. LD₅₀ values and their 95% confidence limits for each treatment were calculated by the Van der Waerden method (see Tables 3.1 and 3.2, below).

Table 3.1: LD50 values and 95% confidence limits for oryzin administered to mice

Administration method	LD50 mg/kg (95% confidence limit)	
	Male	Female
Oral	15,900 (15,000-16,800)	17,300 (16,500-18,200)
Subcutaneous	109.5 (105.4-113.8)	109.5 (105.1-114.2)
Intraperitoneal	64.9 (61.8-68.2)	64.9 (61.8 - 68.2) 64.1 (61.0-67.4)

Table 3.2: LD50 values and 95% confidence limits for oryzin administered to rats

Administration method	LD50 mg/kg (95% confidence limit)	
	Male	Female
Oral	17,800 (17,600-18,000)	14,400 (14,100-14,700)
Subcutaneous	82.9 (73.6-93.5)	103.2 (92.0-115.7)
Intraperitoneal	62.9 (55.8-71.0)	65.3 (57.9-73.6)

Subacute toxicity test (via oral gavage)

The enzyme preparation (as above) was administered to Wistar rats (10/sex/dose) by oral gavage at protease doses of 200, 1000 and 5000 mg/kg bw/day six days per week during a 35 day test period. The control group received the water vehicle only. Animals were weighed and food and water consumption measured every 5 days. Feed (CE-2, NIPPON CLEA) and water were provided *ad libitum*.

No mortality or treatment-related clinical signs were observed during the study period. There were no treatment-related effects on body or body weight gain. No differences between control and treated animals were observed for food and water consumption, organ weights, haematology, or clinical chemistry. Histopathology findings were unremarkable.

Chronic toxicity test

Male Wistar rats (10/dose) were orally administered the enzyme preparation (as above but dissolved in purified water and mixed with powdered feed (CA-1, NIPPON CLEA)) containing protease levels of 0, 500, 1,000 or 2,000 mg/kg bw/day for 26 weeks.

Water was given *ad libitum*. General conditions and food consumption were measured every 7 days. Haematological and urinalysis were carried out at week 12 and 26. Clinical chemistry, macroscopic analysis and histopathological analysis were performed at the end of the test period.

There were no treatment-related deaths. No treatment related clinical signs were observed.

No differences between control and treated animals were observed for food and water consumption, organ weights, haematology, or clinical chemistry. Histopathology findings were unremarkable.

The NOAEL for the enzyme preparation was determined to be 2,000 mg/kg bw/day for male rats.

3.4.2 Genotoxicity

Individual unpublished studies:

Nakayama, M. (1993) Safety assessment of Protease P “Amano” 6 derived from *Aspergillus melleus* – Reverse mutation test in bacteria. Study no. 45-998-05.

Ohta, K. (1994) A chromosomal aberration test of protease P in cultured Chinese hamster cells. Study no. SBL34-00.

The results of these unpublished *in vitro* studies are summarised in Table 3.3. The bacterial reverse mutation tests were conducted with and without metabolic activation (S9) by the pre-incubation test. There was no biologically or statistically significant increases in the number of revertant colonies observed either in the absence or presence of metabolic activation. Positive controls were within expected ranges.

The chromosomal aberration tests involved two experimental approaches. In Experiment 1 the cell cultures were exposed to a range of test article concentrations (see Table 3.3), determined from a dose finding test, in the absence of exogenous metabolic activation (S9) for two time periods (22 h and 46 h). Saline solution used as the negative control and mitomycin C as the positive control. In Experiment 2, the cell cultures were exposed (6 h) to test article (pre-determined concentration range) with and without S9 mix and then incubated for a further 16 h or 40 h. Saline was used as the negative control. Benzo(a)pyrene was the positive control with +S9 and mitomycin C was the positive control with –S9.

In Experiment 1 there was no significant difference in the average frequency of structural aberrations or polyploidy between the treated and the negative control. In Experiment 2 there were no significant differences in the average frequencies of structural aberrations and polyploidy between the treatment groups and the negative control, either with or without S9 mix. In the positive controls the average frequency of structural aberrations was significantly greater than in the negative controls. The frequency of structural aberrations in the negative controls was within acceptable limits.

Based on the above, the applicant concluded that Protease P “Amano” 6 did not induce gene mutations or chromosomal aberrations in these assays.

Table 3.3: Summary of genotoxicity studies

Test	Test system	Test article	Concentration or dose range	Result
Bacterial reverse mutation (Ames test)	<i>Salmonella typhimurium</i> strains TA 98, 100, 1535 & 1537 <i>Escherichia coli</i> strain WP2 <i>uvrA</i>	Protease P “Amano” 6 derived from <i>A. melleus</i> (Lot No. PZQ05520; protease activity: 759,000 µg)	Preliminary Test: (78-5000 µg/plate) Final Test: (313-5000 µg/plate)	Negative (±S9)

Test	Test system	Test article	Concentration or dose range	Result
Chromosomal aberration test	CHL/IU* cells from lungs of new born female Chinese hamsters	Protease P "Amano" 6 derived from <i>A. melleus</i> (Lot No. PZR08524; protease activity: 999,000 µ/g)	Experiment 1 22-hour: 19.5-156.3 µg/mL 46-hour: 4.9-39.1 µg/mL Experiment 2 16 h recovery 78.1-626 µg/ml ±S9 40 h recovery 312.5 – 2,500 µg/mL ±S9	Negative (±S9)

*CHL/IU is a commercially available cell line initiated from Chinese hamster lung fibroblasts

3.4.3 Residual allergens and substances causing food intolerance from the culture medium

Soybean and wheat products (flour and bran) comprise part of the fermentation medium for the preparation of the enzyme. However, residual soy or wheat proteins are not present in the final enzyme preparation (see Section 2.2.1). Dextrin, sourced from potato, is used as the enzyme carrier in the enzyme preparation. All the raw materials used in the production of oryzin are food ingredients that do not raise safety concerns, or food additives permitted in the production of processing aids (FSANZ, 2016).

3.5 Risk Assessment Conclusions

There are no public health and safety issues associated with the use of the product Protease P "Amano" 6, containing the enzyme oryzin (protease) produced by *A. melleus* as a food processing aid on the basis of the following considerations:

- The wild type strain has been characterised as *A. melleus* and is deposited at the Japan Collection of Microorganisms.
- The production organism is not toxigenic or pathogenic. Further, *A. melleus* has a long history of safe use overseas as the production organism for a number of processing aids.
- Residual oryzin is expected to be present in the final food but recommended conditions for use would render the enzyme inactive and it would be susceptible to digestion like any other dietary protein.
- Bioinformatic analysis indicated that oryzin has no biologically relevant homology to known food protein allergens.
- The P "Amano" 6 preparation caused no observable effects at the highest tested doses in a 26-week repeated dose toxicity study in rats. The NOAEL for the oryzin concentrate was determined to be 2000 mg/kg bw/day for male rats.
- The enzyme preparation was not genotoxic *in vitro*

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

References

AMFEP (2015) Association of Manufacturers and Formulators of Enzyme Products, List of Enzymes, <http://www.amfep.org/content/list-enzymes>

ETA (2016) Enzyme Technical Association, Health & Dietary Supplements, http://www.enzymeassociation.org/?page_id=51

FSANZ (2016) Food Standards Australia New Zealand, Food Standards Code, Schedule 15 <http://www.foodstandards.gov.au/code/Pages/default.aspx>

Food Chemicals Codex 9th Edition (2015) The United States Pharmacopeia, United States Pharmacopeial Convention, Rockville, MD. <http://www.usp.org/food-ingredients/food-chemicals-codex>

IUBMB (2016) EC 3.4.21.63 <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/21/63.html>

JECFA (2006) General specifications and considerations for enzyme preparations used in food processing. <http://www.fao.org/docrep/009/a0691e/A0691E03.htm>

Kauffman CA (2006) Fungal infections. Proceedings of the American Thoracic Society 3:35-40.

Matsumura Y (2012) Role of allergen source-derived proteases in sensitization via airway epithelial cells. Journal of Allergy. <http://www.hindawi.com/journals/ja/2012/903659/>

Pariza MW, Johnson, EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regulatory Toxicology and Pharmacology 33:173-186.